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THE DEVELOPMENT OF SPOROZOITES OF *PLASMODIUM GALLINACEUM* INTO CRYPTOZOITES IN TISSUE CULTURE[1]

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Several investigators have reported unsuccessful attempts to transform sporozoites into cryptozoites in tissue culture (Rhodhain, Gavrilov and Cowez 1940; Paraense, Meyer and Menezes 1942; Huff and Coulston 1944; Hawking 1945; Porter 1948). Because of the important implications of such a procedure should it prove successful, another attempt was made. Accordingly, the salivary glands of *Aedes aegypti* infected with *Plasmodium gallinaceum* were inoculated into tissue cultures of normal macrophages from the spleen of chick embryos. The present paper reports the successful development of sporozoites of *P. gallinaceum* into cryptozoites in tissue cultures of normal chicken macrophages.

METHODS AND MATERIALS

A. aegypti mosquitoes infected with *P. gallinaceum* were anesthetized with tobacco smoke or ether. After the wings and legs were removed, the salivary glands were dissected out, using sterile equipment and solutions, and observing sterile precautions. The mosquito was dipped momentarily in 70 per cent ethyl alcohol and then in a balanced salt solution (modified glucosol) containing 100 units of penicillin G (Squibb) and 400 micrograms of dihydrostreptomycin (Squibb) per ml. The salivary glands were dissected out in a drop of the balanced salt solution containing the antibiotics and transferred to another drop of the same solution until five pairs of glands were collected in the latter drop. The five pairs of glands were transferred by means of a dissecting needle into the flask containing the culture of normal macrophages.

The balanced salt solution consisted of sterile glucosol (Parker 1938) which was brought to pH of 7.2 to 7.4 by the addition of a sterile solution of sodium carbonate. Also, this modified glucosol was used to dissolve the antibiotics. The use of the above concentrations of antibiotics was based upon the results of preliminary studies on the effects of penicillin and streptomycin on tissue cultures of macrophages and exoerythrocytic stages of *P. gallinaceum*.

The cultures of normal macrophages were set up four days prior to the inoculation of the sporozoites. The clean cultures were prepared from the spleens of normal 17 to

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19 day-old chick embryos. Cultures were made in bottomless Carrel flasks to which coverslips had been cemented. The details of this procedure will be described elsewhere (Dubin —). The nutrient consisted of 20 per cent chicken serum in Tyrode's solution, with the addition of 0.0025 per cent phenol red as indicator. The pH was controlled by the continuous flow of two per cent CO_2. Just prior to the inoculation of the culture flasks with the salivary glands, fresh nutrient was placed in the flasks similar to that previously used but now containing, in addition, the antibiotics in concentrations equal to those in the modified glucosol. After the macrophage cultures were inoculated with the glands, the flasks were placed in the incubator, connected with the flow of two per cent CO_2 gas mixture, and allowed to incubate at 37°C. for 48 or 72 hours without change of nutrient. The cultures were fixed with absolute methyl alcohol, the cement was removed, and the coverslips stained with dilute Giemsa stain.

Parallel control flasks were set up with salivary glands from non-infected mosquitoes.

RESULTS

In the first experiment six flasks were inoculated with salivary glands and all of these were found to contain typical cryptozoites within the cytoplasm of the macrophages. The control flasks were negative. The appearance of the parasites was identical with that of the exoerythocytic stages of *P. gallinaceum* cultivated from the spleens of infected chick embryos (see figure 1). Only rare clumps of poorly-stained bacteria (presumably dead) were seen. In areas there were faintly-stained sporozoites and debris of salivary glands. In these areas the coverslip was devoid of macrophages, but the macrophages immediately surrounding these sites were infected with cryptozoites. The distribution of the parasites in the coverslip cultures was irregular. Parasitized cells tended to occur in clusters, even up to 150 infected cells in one area; but single infected cells were found scattered throughout the culture. The macrophages contained one to 12 parasites in their cytoplasm.

It was estimated that each culture flask contained about 50,000 to 150,000 macrophages. In the first experiment three flasks were fixed at 48 hours and three at 72 hours. The flasks which were fixed at 48 hours contained respectively 250, 125, and 332 parasitized cells; those that were fixed at 72 hours contained respectively 4, 61, and 13 parasitized cells. The latter flasks had become somewhat acid, as a result of which the cells and parasites stained poorly. This condition probably accounted for the smaller number of parasites in this group.

In the flasks fixed at 48 hours many of the parasites were mature schizonts, with occasional ones almost ready to rupture. The flasks fixed at 72 hours contained parasites which were practically all very young forms, probably metacryptozoites. Also, a few merozoites were seen, lying free in the intercellular spaces or within the cytoplasm of macrophages. In the subsequent experiments, liberated merozoites were seen lying outside cells 48 hours after inoculation of the sporozoites. Thus the developmental cycle from sporozoite to merozoite took approximately 48 hours.

The above results were repeated in subsequent experiments. To date a total of 18

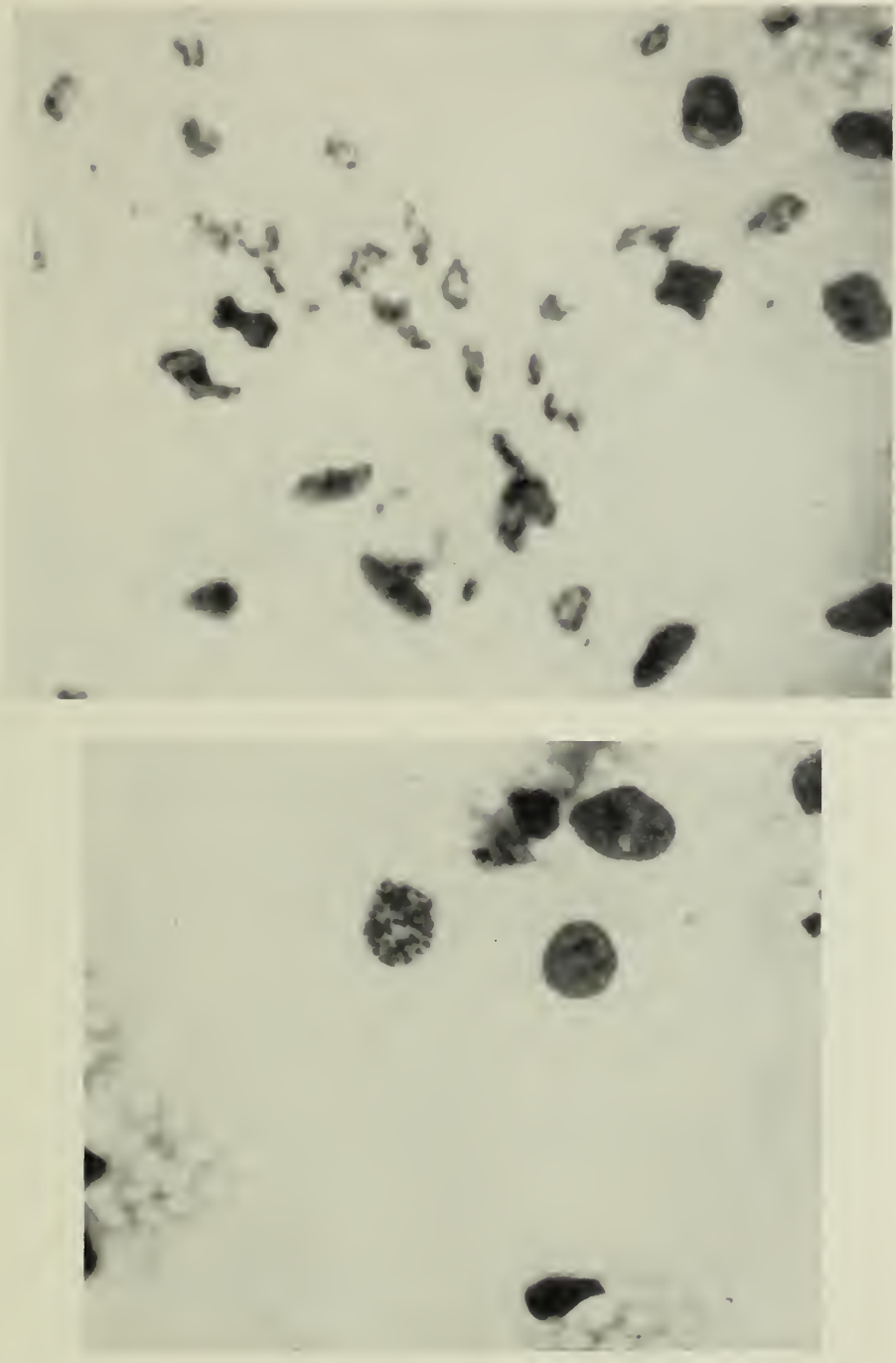

FIG. 1. The upper photomicrograph shows several macrophages containing cryptozoites. There are thirteen parasites in one of the macrophages. The lower photograph shows a binucleate macrophage containing a mature schizont (cryoptozoite stage).

flasks have been so inoculated and of these 12 flasks were positive. Negative results tended to occur in flasks with poor growth of macrophages.

DISCUSSION

The present experiments demonstrate the successful transformation of sporozoites of *P. gallinaceum* into cryptozoites in tissue cultures of normal chicken macrophages.

The senior author attempted a similar experiment three years ago, but failed. At that time he was impressed with two important difficulties: (1) Infection of the cultures arising from the inoculation of the salivary glands and (2) the lack of control of certain environmental factors in the cultures, especially hydrogen-ion concentration. Since that time both penicillin and streptomycin have become readily available and have been used with success in the control of bacterial growth in other types of protozoan cultures. In addition, since that time, the senior author has worked out a simple method whereby the exoerythrocytic stages of *P. gallinaceum* are grown easily and regularly in tissue cultures. For these reasons, another attempt was made at the conversion of sporozoites into cryptozoites *in vitro*.

As a preliminary step the effects of penicillin G and dihydrostreptomycin were studied in tissue cultures of macrophages infected with the exoerythrocytic stages of *P. gallinaceum*. Penicillin had no harmful effect on the host cells or parasites up to 5000 units per ml.; higher dosages were not studied. Dihydrosteptomycin produced a marked damaging effect on macrophages and parasites at a level of 5000 micrograms per ml.; at 1000 micrograms per ml. there was a moderate degree of damage to cells and parasites; at 400 micrograms per ml. the cells were not affected and the parasites were perhaps slightly reduced in number when compared with controls. Accordingly concentrations of penicillin G 100 units and dihydrostreptomycin 400 micrograms per ml. were used. These concentrations proved satisfactory, as evidenced by no bacterial growth in the cultures and no obvious interference with the development of the sporozoites into cryptozoites. Further experiments will have to be done to determine whether lowering the concentration of streptomycin will result in a greater yield of cryptozoites.

Another important problem was to make certain that the bottom of the flask was covered by an adequate number of macrophages in order that there should be an optimum opportunity for phagocytosis of the sporozoites as soon as they reach the bottom of the flask. It was found that merely placing three or four pieces of spleen (each about 1 cu. mm.) on the coverslip, without cementing, resulted in an excellent culture of macrophages in about four days, which almost completely covered the bottom of the flask.

The glands were merely dislodged from the inoculating needle into the nutrient, the glands and sporozoites settling to the bottom of the flask by gravitation. No attempt was made to cement the glands to the coverslip. Further experiments will have to be done to determine what is an adequate number of glands per flask, and to learn the effect of crushing of the glands prior to inoculation.

Huff and Coulston (1944) suggested that the time for complete development of

cryptozoite from sporozoite in chicken tissues was 36 to 48 hours. Our experiments, performed at 37°C., suggest that the time for such a complete development *in vitro* was about 48 hours.

Huff and Coulston (1944) demonstrated the evolution of the sporozoites of *P. gallinaceum* into cryptozoites in chicken tissues and noted that the host cells were macrophages and probably fibroblasts. Hawking (1945) cultivated the exoerythrocytic stages of *P. gallinaceum* in tissue cultures and showed that the host cells were macrophages and elongated cells, which may have been either fibroblasts or reticuloendothelial cells. The senior author demonstrated in tissue cultures that these forms of the parasites grew not only in macrophages, but also in pure cultures of fibroblasts from which macrophages were excluded by using heterologous serum (Dubin ——). The present experiments confirm the above findings and demonstrate that the transformation of sporozoite into cryptozoite can occur in pure cultures of macrophages. It would be interesting to see if such a metamorphosis would occur in pure cultures of fibroblasts.

Further work suggests itself along the following lines: If this procedure can be standardized, an *in vitro* method would be available for the study of the metabolic background for the transformation of sporozoites into cryptozoites, as well as for the study of substances which would inhibit this transformation without undue damage to the host cell. Application to the study of human malaria also suggests itself. At present it is impracticable to obtain for study the exoerythrocytic stages of human malarial parasites. Furthermore, while Shortt and colleagues (Shortt, Garnham, Covell and Shute 1948; Shortt and Garnham 1948) demonstrated that these forms are found in the liver, it is still not clear which cell in the liver serves as the host cell and, it is not known whether the liver is the exclusive site of metamorphosis of sporozoite into cryptozoite. For example, Coulston (1949) reports the finding of cryptozoites of *P. cynomolgi* in the spleen. The success of our experiments suggests that attempts should be made to transform the sporozoites of human malarial parasites into cryptozoites *in vitro*. If successful, this would lead to information concerning the cell or cells in which development of sporozoite to cryptozoite occurs. In addition, it would afford a practicable method for the controlled study *in vitro* of the cryptozoites of human malaria.

SUMMARY

The sporozoites of *Plasmodium gallinaceum* were transformed into cryptozoites *in vitro* by the inoculation of salivary glands of infected *Aedes aegypti* mosquitoes into tissue cultures of normal chicken macrophages.

ACKNOWLEDGMENTS

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MALARIA OBSERVATION STATIONS OF THE PUBLIC HEALTH SERVICE

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Malaria incidence in the United States has declined without significant interruption since 1935 (Figure 1). Reports of locally acquired malaria during the past 15 years also indicate that there has been a considerable diminution in the geographical extent of the disease. The areas where malaria continued to persist, and those from which it has disappeared only recently, have long been recognized as foci of endemic malaria (Figure 2). In these present and former endemic foci, malaria cases, and occasionally deaths attributed to malaria, continue to be reported. While these reports are in many cases questionable, special investigations have disclosed that some of them record bona fide recently acquired malaria infections. (e.g.: Frohne et. al. 1949). Thus, although prevalence of the disease is decreasing, it still remains entrenched in some of the areas where it was formerly endemic.

With the continuing recession of malaria, and its diminishing importance to health organizations, control operations and local surveillance undoubtedly will receive less and less attention. This may result in failure to detect recurring malaria early enough to prevent the disease again reaching epidemic proportions. Cognizant of this probability, the Public Health Service Communicable Disease Center has established a Malaria Investigation Station in each of three areas which are representative of the principal physiographic regions where malaria formerly was highly endemic. Close observations on as many factors as possible which are associated with the natural occurrence of the disease are being made for two principal reasons: (1) To detect as far in advance as possible any indication of an increase or recurrence of malaria, and (2) to find explanations for malaria recession and for renewed transmission, should the downward trend of malaria be reversed. The possibility of renewed transmission cannot be overlooked as long as reasons for the recession of malaria are not understood completely. The concurrent study of past and present conditions related to malaria occurrence may provide more tenable explanations for its precipitous decline than have been proposed. When elucidated, these factors may indicate additional or more effective methods of malaria suppression.

LOCATIONS OF STATIONS

It is postulated that if malaria does tend to increase or to recur, the initial resurgence or reappearance will most likely be in formerly endemic areas. Most malaria in this country has occurred in two general areas: the Mississippi delta from the northern boundary of Arkansas to Natchez, Mississippi, particularly on the alluvial plains associated with tributary rivers, and in the east gulf coastal plain of the southeastern states (Figure 2). One of the "outpost" stations is located in each of these

two areas and a third is located near a large artificial impoundment. The impoundment is under observation as representative of a discontinuous type of malariogenic situation which can be created by man in a variety of physiographic environments.

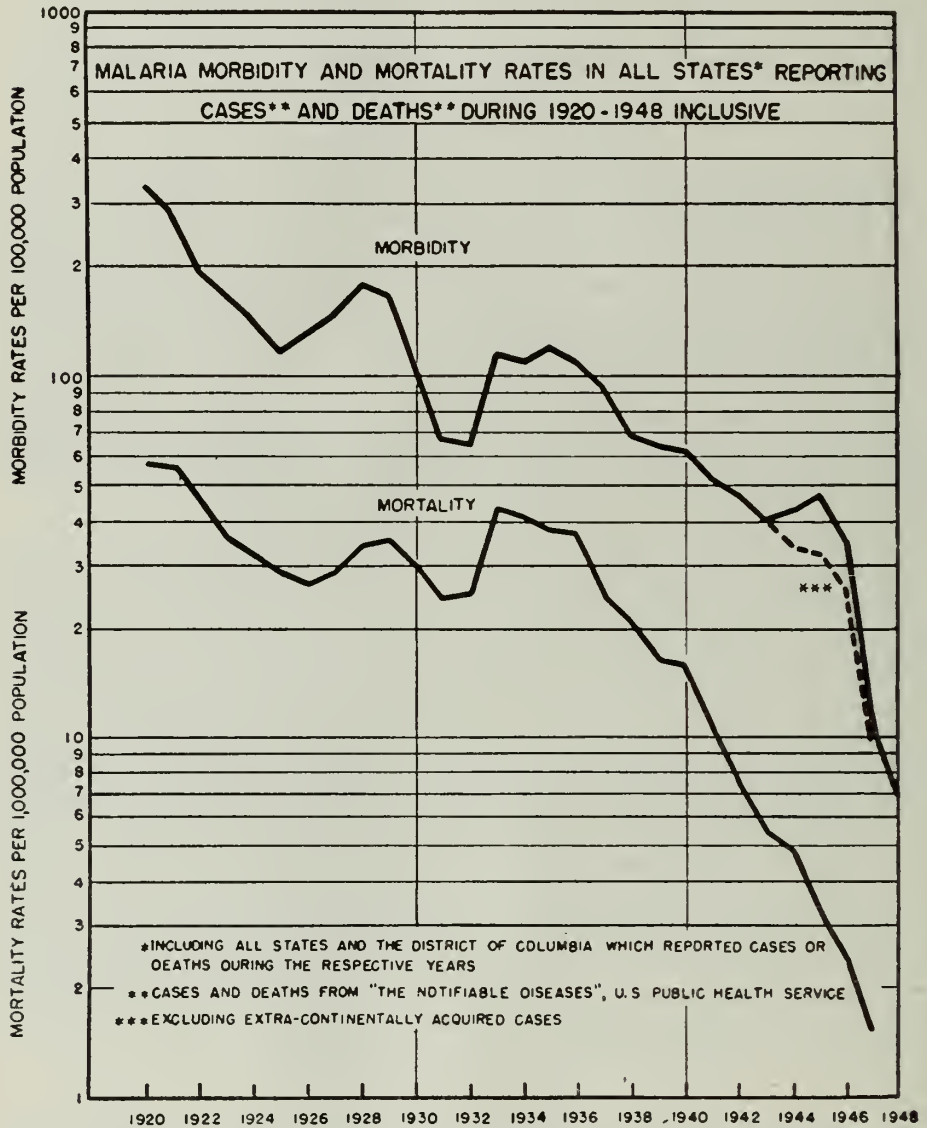

FIGURE 1. Malaria morbidity and mortality rates in all states reporting cases and deaths during 1920–1948 inclusive.

As far as possible, the observation stations were set up in places where facilities were already available and where sufficient local data had been collected to attest the former presence of established endemic malaria. Also, care was taken to select areas

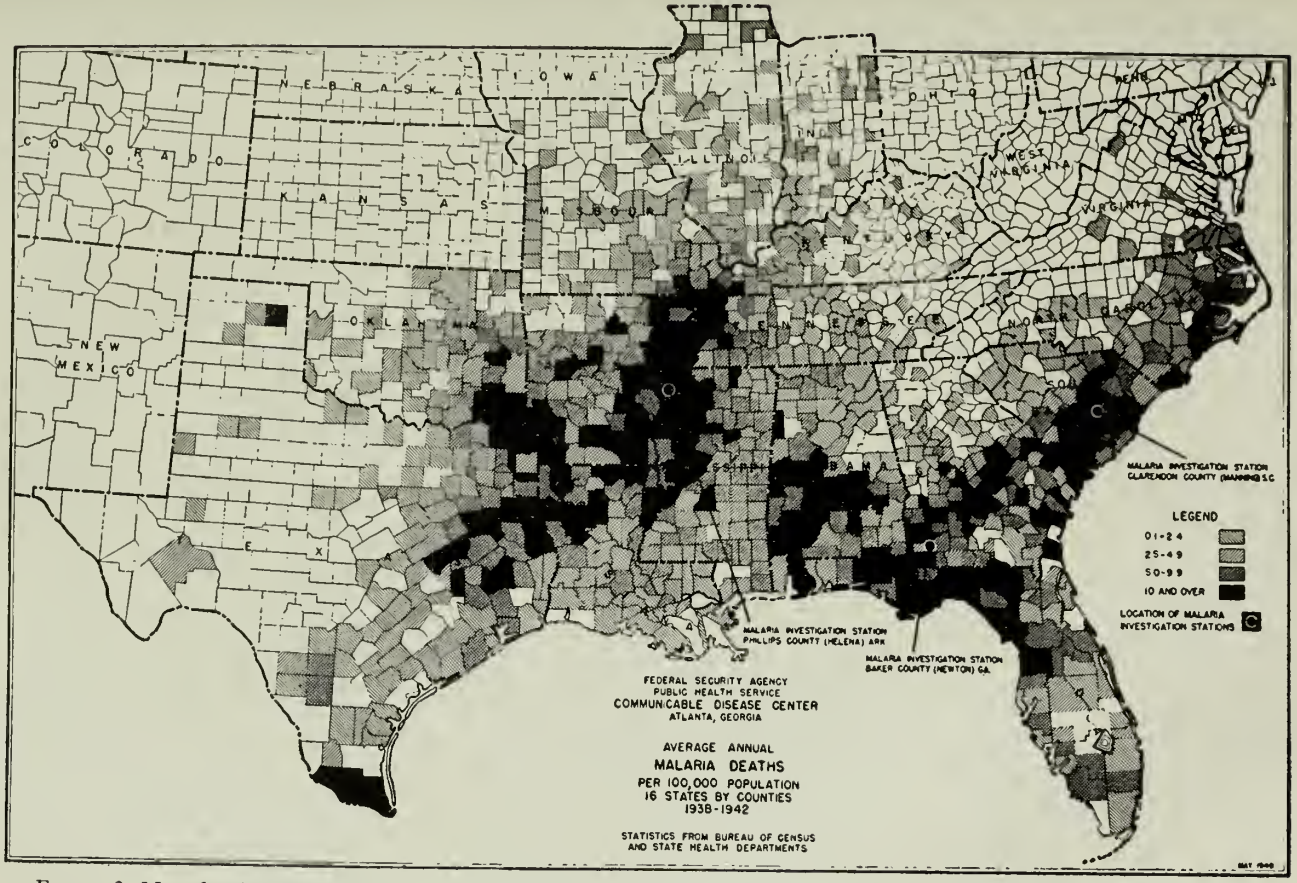

FIGURE 2. Map showing average annual malaria deaths per 100,000 population and locations of Malaria Investigation Stations.

which had undergone no striking change, economic or otherwise, to which local recession of the disease might have been attributed directly.

Mississippi Delta Station. The station in the Mississippi delta was established in the summer of 1948 at Helena in Phillips County, Arkansas. Prior to its recent great recession, malaria in the adjacent sections of Arkansas, Mississippi, and Louisiana was highly endemic. The region constituted one of the heaviest foci of malaria in the United States. Phillips County is representative of this section physiographically and has ranked consistently among the five counties having the highest malaria rates in the state. During the decade 1929 to 1938, malaria mortality rates were higher in Arkansas than in any other state.

East Gulf Coastal Plain Station. The station in the solution topography or limesink region is located in Baker County near Newton, Georgia. This location is characteristic of the coastal plains where numerous water-holding surface depressions, which become conducive to malaria-carrying mosquito production, are formed by subterranean solution. This type of terrain is found principally in parts of South Carolina, Georgia, Florida, and Alabama. The limesink areas are more extensive and have had more malaria than other physiographic types in the United States.

Malaria outpost observations were begun at the Georgia laboratory in 1939 as part of the malaria research program of the Emory University Field Station. Work at the station is now conducted jointly by the Public Health Service and Emory University.

Artificial Impoundment Station. The station for the study of malaria in the environs of an artificial impoundment is located at Manning, South Carolina. It is concerned with malaria conditions in the area contiguous to the Santee-Cooper reservoir. Investigations here were begun by the South Carolina State Board of Health prior to impoundment of the reservoir in 1941 and are now conducted cooperatively by that agency and the Public Health Service.

Programs of Malaria Investigation Stations

The Malaria Investigation Station program is concerned primarily with observation and analysis of factors which contribute directly to the transmission of malaria. The individual stations serve as "lookouts" or "listening posts" in their respective areas. Activities being carried on at present are designed to provide basic information on the following items in each of the study areas:

1. Amount of malaria in man.
2. Relative quantitative index of abundance of *Anopheles* mosquitoes, both larval and adult.
3. Blood feeding habits of local *Anopheles* species.
4. Influence of climatic and other physical factors on *Anopheles* breeding, movement, and longevity.
5. Specific problems of *Anopheles* biology and malaria transmission which are disclosed by the routine observations or which are peculiar to the areas under observation.

Current evaluation of malaria trends is possible from the regular determinations of local malaria prevalence. From these and other data attempts are made to appraise

the possibility of imminent malaria transmission and thus to anticipate future occurrence of the disease. Antecedent information as well as current data on malaria endemicity and associated factors is studied in an effort to evaluate possible causes for the recession in each of the study areas. Special studies are undertaken on problems which arise in connection with the routine observations when necessary to permit effective analysis and interpretation of data related to the over-all program. Several problems related to the natural history of malaria and its vectors are under investigation.

Although the objectives pursued at the three stations are similar, the methods of accomplishment necessarily are adapted to local conditions. This is particularly true of procedures being used for the solution of collateral problems. For this reason separated accounts of each station's activities are presented.

East Gulf Coastal Plains Station.

When the Georgia station was activated in 1939, it was expected that malaria would continue to wane and increase with the apparent rhythmicity which had characterized its course for the previous two decades. The program was projected toward determining what physical or natural factors were associated with these alleged cyclic variations in malaria intensity. Studies were undertaken to (1) appraise the occurrence of human malaria, (2) evaluate densities of *Anopheles*, and (3) study the climatic and hydrological factors obviously related to these measurements.

Measurement of Malaria in Man. During the first year thick blood film surveys were conducted over the entire county (Baker). The results indicated that the majority of local malaria foci were in an area of approximately 40 square miles which included adjoining sections of Baker and Early Counties. After 1939, intensive observations were made in this area and only semiannual blood film surveys were made over the remainder of Baker County, which comprises about 355 square miles and has a population of about 7,000 persons. An experimental area of about 40 square miles was delineated in the section where malaria was most prevalent. The residents, approximately 1,000, were visited regularly by public health nurses experienced in malaria symptomatology in an effort to detect all cases of clinical malaria. When persons with clinical symptoms suggestive of malaria were found, thick blood films were collected. Accurate individual and case history records were kept by the nurses who endeavored to obtain information from affected individuals rather than from an informant for each household.

Figure 3 indicates, by months, the total number of positive blood films obtained in the experimental area. Specimens were collected during surveys, which were designed to provide an adequate sample, and from persons who exhibited clinical symptoms of malaria that were detected in the course of routine visits by the nurses. The yearly figures are considered to be comparable since the same area, and mostly the same population, was involved. A complete analysis of these data is presented elsewhere (Goodwin 1949).

Since it was desired to determine the number of malaria transmissions in the area rather than length of time parasites persist in the blood, all persons with demonstrable parasitemia were treated with quinacrine hydrochloride. Although not intended as a

control measure, the detecting and treating of positive malaria cases must have had a significant effect on control of the disease. It is recognized that similar recessions occurred concurrently in other areas where different types of remedial measures were applied, and even in areas where no control work was done. As yet, no tenable explanation can be offered for the recession of malaria in the study area.

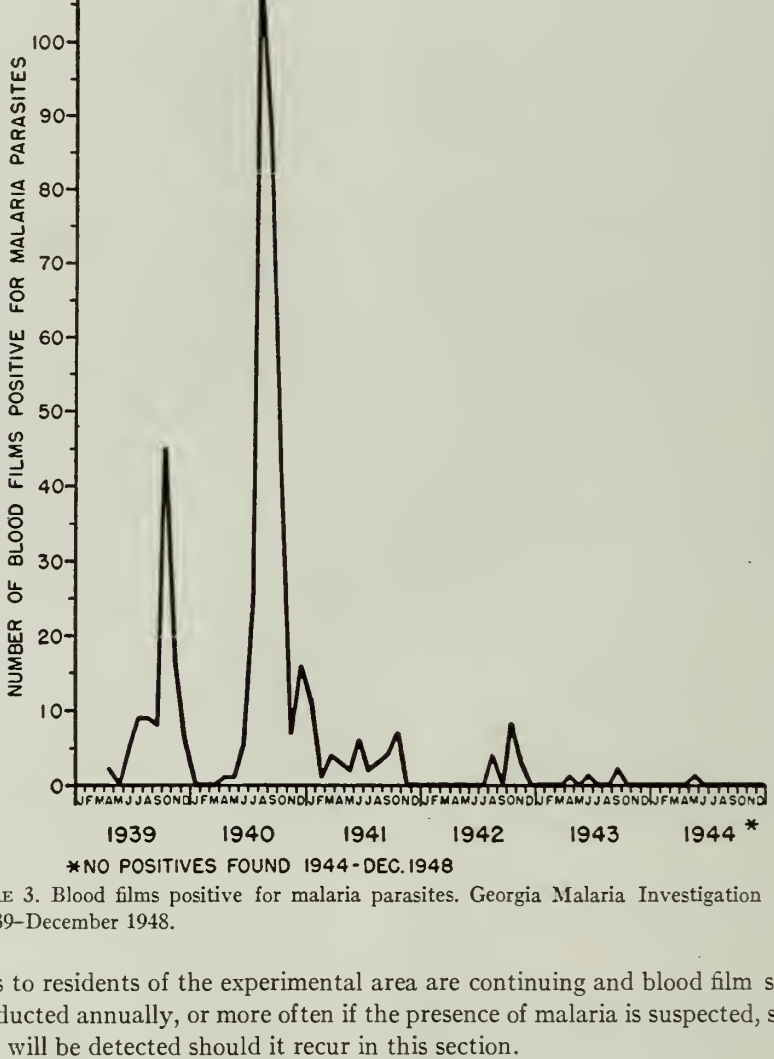

FIGURE 3. Blood films positive for malaria parasites. Georgia Malaria Investigation Station, April 1939–December 1948.

Visits to residents of the experimental area are continuing and blood film surveys are conducted annually, or more often if the presence of malaria is suspected, so that malaria will be detected should it recur in this section.

Studies of Anopheles. Breeding of the three common *Anopheles* species is widespread over the area and a variety of habitats provides suitable conditions for each of the different species. Field data have indicated that no preference is exercised by *quadrimaculatus* in the selection of oviposition sites. The absence of larvae of this species in

some situations is believed to be due rather to unfavorable conditions for larval development. These observations have been confirmed by laboratory experiments (Lund 1942). Studies of flight range of *quadrimaculatus* have shown regular migration from limesink ponds to residences where malaria occurred. The dispersion of this species from breeding areas was also investigated (Goodwin 1949). (In connection with this work a method was devised for marking mosquitoes with fluorescent compounds (Zukel 1945).)

Considerable attention has been given to development of methods for obtaining relative comparable measurements of larval and adult densities (Goodwin 1942; Goodwin and Eyles 1942). Appraisals of larval densities have not been continued but adult indices have been obtained weekly. These measurements, possibly with some modification will continue to be used to detect changes in *Anopheles* populations.

Beginning in 1945, large samples (5000–7000) of *Anopheles* were collected from the experimental area and precipitin tests performed on them to determine sources of blood meals. Results of these examinations indicate a great preponderance of equine, bovine, and porcine feedings; less than 0.1 per cent of the specimens tested had fed on man. This very low human feeding rate may be significant, and the continuation of these tests is planned so that any change in this phenomenon will be detected.

Studies on winter-resting places and winter physiology of *Anopheles* (Zukel 1949a, b) have shown that *Anopheles quadrimaculatus* mosquitoes do not actually hibernate in the South Georgia area but that the females survive the cold months and become active during warm periods of the winter. Indications were that oviposition occurs and larval development proceeds at a greatly reduced rate in the winter. Water temperature appears to be the determining factor in larval development and air temperatures have been analyzed in an effort to determine more exact relations of these factors to mosquito activity and also, to establish relations between water and atmospheric temperatures (Hendricks and Goodwin 1949).

Climatic and Hydrological Studies. These studies were undertaken to determine the relation between malaria intensity, *Anopheles* production, pond levels, rainfall, and other physical factors. The disappearance of malaria from the area precluded consummation of this objective. It was possible, however, to appraise the relation of various physical factors to the occurrence of *Anopheles*-producing ponds. General methods for this study have been presented (Goodwin and Lenert 1943). These hydrological studies were conducted in cooperation with the Water Resources Branch of the U. S. Geological Survey. Detailed accounts of the physiography of the region and hydrological characteristics of limesink ponds are to be given elsewhere (Hendricks 1949a, b).

Artificial Impoundment Station.

Areas of Clarendon, Berkeley, and Orangeburg Counties, South Carolina, along the Santee River have long histories of intense malariousness. Construction of a reservoir for hydroelectric development in 1941 intensified the local occurrence of malaria. Studies of the problem had been made periodically by the South Carolina State Board of Health prior to 1939, but in that year systematic entomological and epi-

demiological observations were started. The Santee Cooper Survey of 1944 conducted by the Public Health Service and the state provided a detailed appraisal of conditions then existing and furnished much additional information on malaria in the region (Anonymous 1946; Link 1947). Since that time, observations designed to utilize and supplement the data obtained previously have been continued. Studies in this

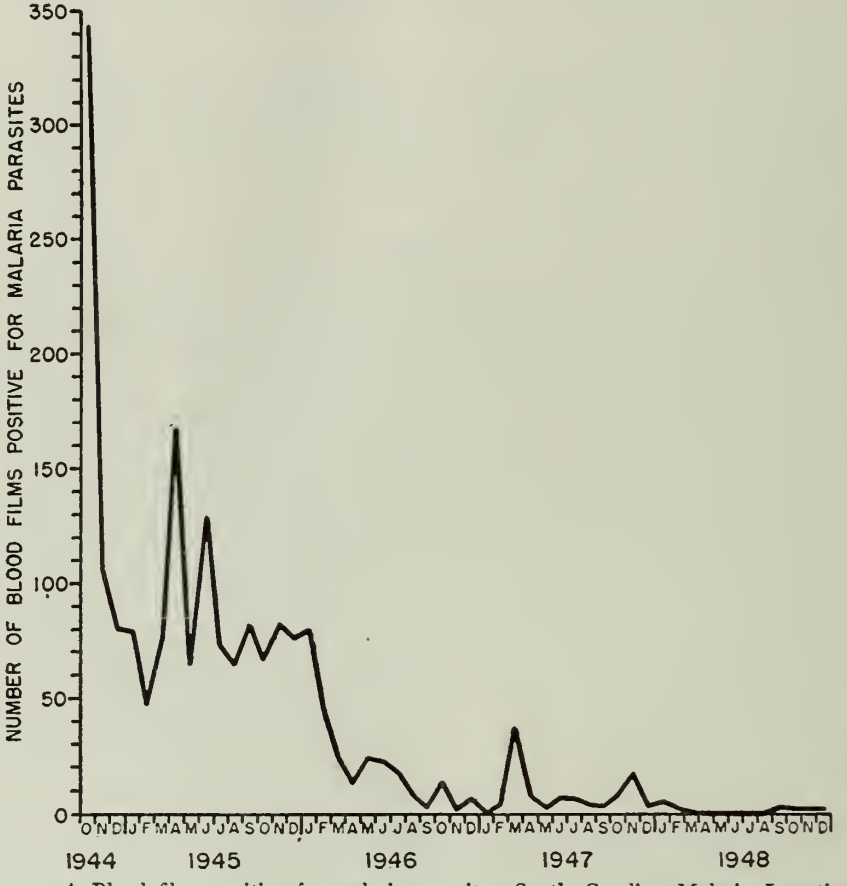

FIGURE 4. Blood films positive for malaria parasites. South Carolina Malaria Investigation Station, October 1944–December 1948.

area are of particular significance since the only known demonstrable focus of endemic malaria in the country at present exists here.

Malaria Morbidity Measurements. Blood film surveys made in 1944 indicated that malaria was most prevalent in a section of Clarendon County adjacent to a tributary of the reservoir called Potato Creek. Subsequently, intensive observations were conducted in this section, and in one area thick blood films have been obtained monthly since 1944 from virtually all of the 2,000 residents. Quarterly surveys, involving about 2,000 additional persons, were conducted in marginal areas adjacent to the intensive area. Figure 4 shows the number of positive blood films found by the monthly and quarterly surveys of this population. Nearly 20 percent of the population

in this section had positive blood films in October 1944. Positive blood films are now encountered only rarely. However, certain of these can be explained only on the basis that malaria is still being transmitted to some extent in the area.

The monthly surveys have been highly effective in detecting persistent malaria infections. Since positive cases were not treated it was possible to follow relapses in a number of individuals over a period of several months. These studies have provided evidence which indicates unusual relapse rates for *Plasmodium falciparum* (Reider and McDaniel 1946). The persistence of *malariae* infections for long periods has also been noted. The general area is known to contain a focus of *malariae* (McDaniel and Hemphill 1948) and study of the occurrence of this infrequently found parasite has been undertaken.

Studies of Anopheles. In the Santee Cooper area, barns have been selected as the type of adult anopheline resting place most suitable for use in making routine measurements, since they are generally of the same construction and appear to be comparable in other respects. Regular weekly inspections are made for enumerative purposes and to obtain mosquitoes for dissection and precipitin tests.

Routine larval collections also have been made from selected stations in the reservoir, including several at some distance from the shore wherein prolific breeding occurred. To determine if adult mosquitoes produced in these areas were migrating to the shores, marked mosquitoes were released in the center of the reservoir. The subsequent recapture of marked specimens in the marginal zones showed that under the conditions prevailing flights of more than two miles could occur (Eyles, et. al. 1945).

Precipitin tests to determine feeding habits of the *Anopheles* have given findings which parallel those of the Georgia station. Samples of *crucians* and *quadrimaculatus* have been dissected at approximately weekly intervals since the fall of 1944. Gland infections continue to be found in both species (Sabrosky, et. al. 1946). Because of the unusual finding of numerous sporozoite-positive *crucians* in nature, special studies were undertaken to determine if the sporozoites were from an infection in man or in a lower animal. In pursuing this problem a limited number of parasitological examinations have been made of the blood of birds and other vertebrates (Hart 1949). As yet no suggestion as to the identity of the sporozoites has been obtained.

Data from dissections and *Anopheles* abundance measurements have been evaluated to determine the relation of infectiveness and density of *quadrimaculatus* (Weathersbee and Frohne 1948). The persistence of gland infections in recognized human-malaria vectors suggests that some malaria is still being transmitted in the area (Frohne, et. al. 1949). The incrimination of *crucians* as a vector of a *Plasmodium* species has stimulated investigations on the biology of this mosquito, which heretofore has received little attention. The first report of this work, concerning winter habits, has been presented (Frohne and Hart 1949).

Mississippi Delta Station.

The program inaugurated recently at the Helena, Arkansas station is essentially similar to those of the other two stations. Collections of data have not been conducted for a sufficient period to permit summations or analyses.

SUMMARY

The Public Health Service has established malaria observation stations in the three principal types of traditionally endemic malaria areas of the southeastern states. Observations are designed to permit prompt recognition of any indication of malaria resurgence. Activities at these stations include observation of factors which cause or are related to the occurrence of human malaria. Current appraisals are made of the malaria-transmission potential. Investigations are made on the natural history of malaria and on the biology of malaria vectors.

Stations are located (1) on the Mississippi delta at Helena (Phillips County), Arkansas, (2) near a large artificial impoundment at Manning (Clarendon County), South Carolina, and (3) in the limesink area of the southeast near Newton (Baker County), Georgia.

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OBSERVATIONS ON DISPERSAL OF *ANOPHELES QUADRIMACULATUS* SAY FROM A BREEDING AREA

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The studies reported here were undertaken to determine whether *Anopheles quadrimaculatus* disperses quickly from breeding places or tends to accumulate and remain in the vicinity of these areas. Most previous studies of the flight habits of *Anopheles* species were performed to determine range of flight rather than to obtain information on dissemination of the adult mosquitoes from a specific location. (Eyles (1944) presented a review of the literature on flight range and dispersion of *Anopheles*.)

Barber and Hayne (1924) made observations on the dispersal of adult *A. quadrimaculatus* from natural resting places near residences in the vicinity of Stuttgart, Arkansas. Mosquitoes were stained by spraying individuals in the resting places with aqueous solutions of aniline dyes. The percentage of stained specimens found subsequently in random samples from the same stations was determined. In two tests 26.9 per cent and 35.7 per cent respectively were found stained the first day following staining. This expression of results provides information on the ratio of marked to unmarked specimens at the time of collection, but does not indicate the per cent of stained specimens recovered. From the data given in their report it is calculated that approximately 11.5 per cent and 2.0 per cent respectively of the samples marked originally were recovered. In one instance it was observed that about one-fourth of the mosquitoes in the shelter during the day remain after the initial exodus at dusk. It was also found that no appreciable accumulation of adult *A. quadrimaculatus* occurred in the resting places examined. After the first day the number of marked specimens dropped rapidly. Only 1.2 per cent of the number stained were recovered on the second day following and after the sixth day none was collected. The data presented indicate that some individuals remained for a time in the resting place. Presumably these were engorged specimens which were digesting blood and developing ova. Kumm (1929), working in North Carolina, found that *A. quadrimaculatus* adults seldom remained for longer than 24 hours in resting places where they probably obtained a blood meal. In his experiments, 23 specimens of 616 stained were recaptured. Fifteen of the marked specimens were collected on the first day after staining and 10 of these, 1.6 per cent of the number marked, were collected from the same places in which they were stained. Of all the marked mosquitoes recaptured, 12, 1.9 per cent of the total number marked, were taken from the place where they were stained.

There may be many unrecognized factors which influence nocturnal dispersion of

adult mosquitoes. However, the stimulus for flight to the types of resting places studied by Barber and Hayne and by Kumm must be the urge to feed. Flight away from them must be for the purpose of oviposition. It is likely that mosquitoes which are in resting places near breeding areas are newly emerged specimens, females which are seeking an oviposition site, or males which tend to remain near the place where they emerge. Thus, the behavior of adult mosquitoes in the environs of breeding places would be expected to differ from that near sources of blood meals since the stimuli for flight are different. In the two studies mentioned above observations were made of movement of mosquitoes near sources of blood; the observations described below were made on *A. quadrimaculatus* near a prolific breeding area.

PROCEDURE

Specimens used in the present experiments were obtained from natural resting places and from one foot cubical red boxes (Goodwin 1942) on the margin of a limesink pond which produced each year a large population of *A. quadrimaculatus*. Adult insects were collected in aspirator tubes, counted, and placed in a screened cage. When as many specimens were collected as possible, the mosquitoes inside the cage were sprayed with an atomized solution of dye. In two cases 1 per cent aqueous solution of gentian violet was used and in a third experiment 1 per cent saffarin A was used. Specimens were released immediately after spraying. The cage was put in a shaded location near an adult mosquito resting place and left open for a period of two hours or longer. During this time most of the mosquitoes left of their own accord; the remainder were shaken out of the cage. It was determined that the mortality resulting immediately from spraying and handling was less than 0.1 per cent. Examination of a sample of specimens in the cage indicated that virtually all were stained. In one case mosquitoes were collected from the resting place immediately adjacent to the point of release two hours after release. Of this number, 90 per cent were found to be stained. Recent emergence may have accounted for the unstained individuals.

Collections were made on subsequent days from the same stations from which the original lot was obtained. Mosquitoes were killed in chloroform tubes, placed in ointment tins, and transported to the laboratory. Individual specimens were placed in porcelain depression plates and a few drops of destaining solution composed of equal parts of glycerin and alcohol were placed on each specimen. After 15 minutes the plates were examined macroscopically for evidence of stain.

RESULTS

Results of three tests are shown in the accompanying tables. In the first test, 4.2 per cent of 2,352 specimens stained were recovered on the first day after staining, 1.9 per cent on the second day, and 1.2 per cent on the third (Table 2). On the first three days following staining 7.4 per cent of the marked mosquitoes were recovered. In all examinations subsequent to release 7.6 per cent of the stained *A. quadrimaculatus* were found. In collecting specimens for staining it was impractical to determine sex ratio. Based on relative numbers in subsequent captures this ratio appears to be 2 males to 1 female (67 per cent males). The ratio of marked males to

marked females was 1.35:1 on the first day after staining, 4:1 on the second, and 6.25:1 on the third. The per cent of all males and females recovered from the original lot marked was approximately the same, however (Table 2). On the first day after staining a greater proportion of marked females than marked males was found. In

TABLE 1

Number of specimens examined, number found stained, and per cent of collection stained

DATE 1944	DAYS AFTER STAINING	NUMBER EXAMINED			STAINED SPECIMENS					
					Number			Per cent of collection		
		M	F	T	M	F	T	M	F	T
Experiment 1, 3 July 1944, 2352 specimens stained with gentian violet and released										
4 July	1	1667	661	2328	57	42	99	3.4	6.4	4.2
5 July	2	1274	395	1669	36	9	45	2.8	2.3	2.7
6 July	3	1143	507	1650	25	4	29	2.2	0.8	1.8
13 July	10	1169	442	1611	1		1	0.1		
18 July	15	1054	626	1680	2	3	5	0.2	0.5	0.3
21 July	18	1023	797	1820	1		1	0.1		
27 July	24	948	661	1609						
3 Aug.	31	1081	454	1535						
10 Aug.	38	1164	570	1734						
20 Sept.	79	564	383	947						
Total.....		11,087	5,496	16,583	122	58	180	1.1	1.1	1.1
Experiment 2, 17 July 1944, 1705 specimens stained with saffarin A and released										
18 July	1	1054	626	1680	45	18	63	4.3	2.8	3.7
21 July	4	1023	797	1820	9	2	11	0.9	0.2	0.6
27 July	10	948	661	1609	1	2	3	0.1	0.3	0.2
3 Aug.	17	1081	454	1535	1		1	0.1		
10 Aug.	24	1164	570	1734						
20 Sept.	65	564	383	947						
22 Sept.	67	454	384	838						
28 Sept.	73	558	497	1055						
Total.....		6,846	4,372	11,218	56	22	78	0.8	0.5	0.7
Experiment 3, 21 September 1944, 1094 specimens stained with gentian violet and released										
22 Sept.	1	454	384	838	17	21	38	3.7	5.5	4.5
28 Sept.	7	558	497	1055	3	2	5	0.5	0.4	0.5
Total.....		1,012	881	1,893	20	23	43	2.0	2.6	2.3

the second and third collections the proportion of males was larger. The numbers of stained specimens in the other collections were too small for valid comparison (Table 2). The per cent of marked specimens in collections made following staining, i.e. number found stained divided by number collected was 3.4 per cent males, 6.4 per cent females on the first day; 2.8 per cent males, 2.3 per cent females on the second

day; and 2.2 per cent males, 0.8 per cent females on the third. One stained male specimen was found 18 days after release (Table 1).

Results of the second test parallel those of the first. On the first day following staining, 3.7 per cent of 1,705 specimens marked were recovered; on the fourth 0.6 per cent were found. In all 4.6 per cent of the lot stained was later detected (Table 2). The smaller percentage of marked individuals found is probably due to the absence of observation on the second and third days after staining. In the first two collections in this test the percentage of females found from the stained lot was lower than the percentage of males; in the third the percentage of females was higher,

TABLE 2

Percentage of stained specimens recovered, by sexes, in collections made at intervals subsequent to release

NUMBER OF DAYS AFTER STAINING	EXPERIMENT NO.								
	1			2			3		
	Males	Females	Total	Males	Females	Total	Males	Females	Total
	Number stained								
	1575	777	2352	1040	665	1705	580	514	1094
	Per cent of stained specimens recovered								
1	3.6	5.4	4.2	4.3	2.7	3.7	2.9	4.1	3.5
2	2.3	1.2	1.9			*			*
3	1.6	0.5	1.2			*			*
4				0.9	0.3	0.6			*
7			*			*	0.5	0.4	0.5
10	†		†	0.1	0.3	0.2			*
15	0.1	0.4	0.2			*			*
17			*	†		†			*
18	†		†			*			*
Total all days.....	7.7	7.5	7.6	5.4	3.3	4.6	3.4	4.5	3.9

* indicates that no observation was made.

† indicates a single specimen collected which represented less than 0.1% of original lot stained.

number of each sex calculated from the total number of specimens stained on the basis of ratio of sexes observed in subsequent collections.

but the small number of specimens involved may account for this divergent result. The ratio of marked males to females was 2.5:1 on the first day and 4.5:1 on the fourth day. The ratio of males to females during this experiment was approximately 1.6:1 (61 per cent males). This deviation from observation in the first test may have been due to a lull in breeding or to conditions adverse to longevity of males. The percentage of marked males and females in collections was 4.3 per cent males, 2.8 per cent females on the first day and 0.9 per cent males, 0.2 per cent females on the fourth. A single marked male was collected 17 days after staining (Table 1).

Data obtained during the third test are meager but, in general, they follow the pattern of the previous ones. At the time observations were made, the ratio of males to females was about equal (53 per cent males). The per cent of marked specimens

detected on the first day following staining was 3.5 per cent of 1,094; on the seventh 0.5 per cent (Table 2). In the first collection 3.7 per cent of the males and 5.46 per cent of the females collected were marked and on the seventh day 0.5 per cent of the males and 0.4 per cent of the females showed evidence of dye (Table 1). As in the first test, the per cent of females recovered from the original sample on the first day after staining was greater than the per cent of males. In the only other collection of this series the per cent of males was slightly higher.

DISCUSSION

Although the data accumulated are not sufficient to justify generalizations, some inferences can be made. In two of the tests, the proportion of marked *A. quadrimaculatus* females recovered on the day after release was greater than the proportion of males in the sample stained. This suggests that females may, under some conditions, disperse from the breeding area later than do males. The females leave the breeding area soon, however, as indicated by the comparatively small per cent found in collections made following the first day after staining. Female specimens near breeding places are most likely newly emerged individuals or engorged ones which have returned to a water area to oviposit. The high proportion of females found soon after staining is probably due to the fact that they remain in the vicinity to mate or oviposit.

Barber and Hayne (1924) showed that the dispersal of females from resting places near sources of blood is much slower than that observed here from resting places near the breeding area. They collected some individuals as long as six days after marking. This does suggest that adults remain in the shelter for several days, as they propose. However, when they confined adults in the resting places by screening all exits, very few survived for as long as six days. It is more likely that the marked individuals found on successive nights reentered the same resting place by sheer chance after the random sorting of the population each night. That this type of dispersal does occur is shown by the data presented here. The finding of stained mosquitoes repeatedly on consecutive nights in the places from which all specimens had been collected indicates a thorough mixing of the *A. quadrimaculatus* in the area. Only rarely were any mosquitoes observed after dark in resting places near breeding areas. The occasional individuals noted are believed to have been ones which had taken a blood meal in the immediate vicinity. Virtually all *A. quadrimaculatus* in diurnal shelters at the breeding place are active during the night.

It is commonly recognized that male *A. quadrimaculatus* exhibit a greater tendency to remain near the breeding area than do females. This is shown by the ratio of males to females in collections following staining, the per cent of marked specimens in these collections, and by the fact that males in collections made near breeding places greatly outnumber females, although the sexes are produced in about equal number. In these tests, however, approximately the same proportion of males and females as were in the original sample were recovered. This indicates that the dispersal of males is as general as that of females. Although there is good evidence that sorting is limited to the population in the immediate environs of the breeding area, a few males are found far from breeding areas in resting places frequented by freshly en-

gorged females. The longevity of males is attested by the recovery of one on the seventeenth and one on the eighteenth day after staining.

SUMMARY

Three lots of *Anopheles quadrimaculatus* adults were collected from resting places near a prolific breeding area, stained with aqueous solutions of aniline dyes, and released. Of 18,476 specimens examined subsequently, 301 or 1.6 per cent were found stained. Of the 5,151 mosquitoes stained originally 5.8 per cent were recovered.

Females tend to remain close to the breeding area for a short time, probably to mate or oviposit. On the first day following staining a larger proportion of marked females than marked males are found. After the first day this proportion is reversed.

Male adults were found in greater numbers at the breeding place than were females. There is evidence that males disseminate as generally as females but only over a limited range near the breeding area. In two instances a single marked male was found after females were no longer collected; in one case 17 days after staining and in the other 18 days.

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ROUTINE CULTURE METHODS IN DIAGNOSING *ENDAMOEBA HISTOLYTICA*

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Many publications have dealt with the problem of laboratory diagnosis of intestinal parasites, especially the ameba. In spite of this fact the clinical laboratory diagnosis of amebiasis remains in a chaotic condition. Rentrop and Tribby, (1949) in a survey of a group of commercial and hospital laboratories found that suspected amebiasis cases were being reported as positive for *Endamoeba histolytica* in the stools from 0 to 72.9 per cent. The authors believe this is due in part to the lack of technicians trained in the recognition of the parasites and the reluctance on the part of the laboratories to adopt improved techniques.

Many authorities differ in their opinion as to the practical value of cultural methods in the laboratory diagnosis of *E. histolytica*. However, as more information accumulates the evidence seems to indicate that a culture method might well be adopted as one of the standard procedures of diagnosing amebiasis. Craig and St. John (1927) found that cultural methods were superior to either the sedimentation method or the direct examination when only one specimen was examined. Tsuchiya (1942), Balamuth and Howard (1946) and others strongly advocate the use of cultural methods as a diagnostic procedure. On the other hand there are authorities who maintain that other diagnostic procedures are better and most clinical laboratories cling to the method of examining only fresh saline preparations of purged stools.

The purpose of this paper is to present data comparing four culture methods of laboratory diagnosis with the Heidenhain's iron-hematoxylin stain method. This study was undertaken on the request and with the support of a group of physicians practicing in Memphis, Tennessee.

MATERIALS AND METHODS

Stool specimens were collected and brought to the laboratory as soon as possible, most of them arriving within four hours after being passed. Smears were made immediately and placed in Schaudinn's fixative for staining by the iron-hematoxylin method. Fresh saline and iodine preparations were then examined and a portion of the specimen was introduced into culture medium. An effort was made to use two or more culture media on each specimen, however in 117 of 350 specimens only one medium was used.

Nelson's egg yolk alcoholic extract medium (1947), St. John's beef heart extract medium (1932), Balamuth's medium special[1] and an egg slant overlayed with buffered saline (Shaffer and Frye, 1948) were the media used in this study.

[1] Furnished by Difco Laboratories, Detroit, Michigan.

RESULTS

Since the stool specimens were unpurged, few parasites were observed in the fresh saline or iodine stained preparations. An occasional cyst was found in the iodine preparations. In the more acute cases of amebiasis trophozoites were observed in the saline preparations. Since we were interested in finding the chronic cases as well as the acute we will confine our discussion to the results obtained in the culture and iron-hematoxylin stain methods.

Because intestinal protozoan parasites, especially in chronic cases and carriers, are not demonstrable in every stool specimen, each patient was asked to submit

TABLE 1

Number of specimen on which first positive for E. histolytica *was found in 145 patients examined*

	SPECIMEN NUMBER			TOTAL	PER CENT OF TOTAL PATIENTS
	1	2	3		
Positive by culture method, Nelson's	18	6	1	25	17.2
Positive by I. H. Stain method	12	7	1	20	13.8
Positive by both methods	18	7	1	26	17.9

TABLE 2

Summary of data showing number of specimens positive for Endamoeba histolytica *in four culture media compared with iron-hematoxylin stain*

NO. OF SPECIMENS	NO. OF POSITIVE SPECIMENS				
	Nelson's	St. John's	Egg Slant	Balamuth's	I. H. Stain
22	2	0	—	—	1
48	5	2	1	—	3
71	12	—	9	—	8
90	10	—	9	8	7
117	19	—	—	—	9
2	0	—	—	0	0
350	48 (350)	2 (70)	19 (209)	8 (92)	28 (350)

Total specimens examined are in parentheses.

three stools with a day intervening between specimens. From 145 patients we obtained an average of only 2.4 stools per patient. Ninety-seven of the 145 patients submitted three or more stools, 11 patients submitted two specimens and 37 submitted only one. From these 145 patients there were 26 (17.9 per cent) found to harbor *E. histolytica*. It will be found by examination of table 1 that, although most of those harboring *E. histolytica* were discovered on examination of the first specimen submitted, seven were found in the second specimen and one in the third.

In order to find a culture medium suitable for isolation and in which strains of the parasites could be maintained in culture, the four media mentioned above seemed to be the most promising with which to start this study. Of the 26 patients found to be positive for *E. histolytica*, 25 (17.2 per cent of the total patients studied) were positive

in culture while only 20 (13.8 per cent) were positive by the iron-hematoxylin stain method. Some of the latter had so few parasites that they were revealed only after prolonged study of the stained smear.

Table 2 gives the data collected on the 350 specimens obtained from the patients under study. There were 48 specimens which gave a positive culture for *E. histolytica* while only 28 were found to be positive with the iron-hematoxylin stain method. There were however two specimens found positive on iron-hematoxylin that were not found to be positive in culture. There were then, a total of 50 (14.3 per cent) of the 350 specimens found positive by using both methods. None of the other culture media revealed the presence of *E. histolytica* which was not also found to be present in Nelson's medium. Of 7 positive specimens cultured in St. John's medium only two showed any growth. Of 27 positive specimens only 18 showed any growth in the egg slant and of 10 positive specimens 8 grew in Balamuth's medium special. There were 29 of these specimens all of which produced good growth in Nelson's medium.

TABLE 3

Number of patients harboring parasites other than Endamoeba histolytica

PARASITE	NO. OF PATIENTS
Endamoeba coli	32
Endamoeba nana	21
Iodamoeba butschlii	1
Giardia lamblia	2
Trichominis hominis	2
Chilomastix mesnili	10
Strongyloides stercoralis	1

Nelson's medium proved to be good not only as a diagnostic medium but also as medium in which to carry strains of *E. histolytica*. We were able to confirm Nelson's observation that transfers need not be made at frequent intervals. Transfers can be made at 4–5 day or longer intervals and the strain will continue to grow well. One strain was subcultured after 18 days without noticeable damage to the ability of the organism to reproduce. This strain has since been subcultured 23 times. We have carried 13 strains at least four passages, one having gone through 36 passages. Ameba other than *E. histolytica* tend to die out rapidly and will not persist for more than about two passages of the culture. This medium was also used for *in vitro* tests on the amebicidal activity of aureomycin (McVay, Laird and Sprunt, 1949).

Another incidental observation was made on two stools containing large numbers of *E. histolytica* cysts. The specimens were kept in the refrigerator, periodically removed and a small amount of feces introduced into Nelson's medium. These cysts remained viable and produced good cultures up to 21 days in one case and 27 days in the other.

Seven of the 26 patients positive for *E. histolytica* did not have any other parasite, the remaining 19 had one or more of the other intestinal parasites. Table 3 lists the parasites other than *E. histolytica* and gives the number of patients harboring each.

DISCUSSION

The authors agree with Nelson in that the alcoholic extract cultivation medium is a ". . .practical and effective medium for the diagnosis of *Endamoeba histolytica.*" This is a simple medium to prepare and it keeps well under refrigeration. Very often a luxuriant growth of ameba is produced in 24 hours even from stools with extremely few parasites. As has been emphasized by Tsuchiya (1942) and others the skill of the microscopist is still depended upon for the final identification of the organism. Although final identification in this study was made by use of the polyvinyl alcohol fixative technique of Goldman (1948), in working with the cultures one soon learns to make a differential diagnosis without the aid of stain.

SUMMARY

Data has been presented comparing the diagnostic value of four different media with the Heidenhain's iron-hematoxylin staining method. The data confirms the opinion of others that the use of a good culture medium should be added as a routine procedure in the laboratory diagnosis of *E. histolytica.*

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PARASITES FOUND IN CERTAIN SCIURIDAE OF THE SOUTHWESTERN UNITED STATES

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The finding of malaria in the Malabar squirrel by Mulligan and Sommerville (1947) confirmed the original report of Donovan (1920) and gave added impetus to the search for a small laboratory mammal which could be infected with one of the strains of plasmodia. Since it was not possible to import infected Malabar squirrels (*Sciurus indicus malabaricus*) from India, the possibility that a natural infection with plasmodia might occur in some of the Sciuridae of the United States was investigated.

METHODS

It was necessary to select an area where the work could be conducted with minimum expense and where the geographic and climatic conditions make transmission possible. The U. S. Public Health Service Laboratory in San Francisco has for several years conducted plague surveys of the indigenous rodent population in numerous areas in the Western United States. Arrangements were made for one of the authors to accompany a survey team throughout May and June and again for one week during September of 1948 during its operation in Southwest Texas and Southeast Oklahoma. Thick blood films made on all animals killed or captured were stained with Giemsa stain at the end of each work day and were mailed to the Memphis Laboratory for examination.

RESULTS

Examinations were made of blood films from 140 fox squirrels (*Sciurus niger* ssp.), 29 gray squirrels (*Sciurus carolinensis* ssp.), 75 black-backed rock squirrels (*Citellus variegatus buckleyi*), 3 Say's rock squirrels (*Citellus variegatus grammurus*) 28 Rio Grande ground squirrels (*Citellus mexicanus parvidens*), and 37 prairie dogs (*Cynomys ludovicianus* ssp.). Of these 312 rodents, 13 black-backed rock squirrels, 2 fox squirrels and one Rio Grande ground squirrel were captured alive and brought to the laboratory for further study. Twenty-nine gray squirrels and five fox squirrels (probably *Sciurus niger rufiventer*) were obtained in Pushmataha County, Oklahoma while the remaining 278 animals came from Zavala, Uvalde, Kimble, Menard and Valverde Counties in Texas.

While the survey failed in its original purpose, since plasmodia were not found, blood films from 20 per cent of the rodents examined exhibited an interesting parasite population. The parasites found, a piroplasm, a trypanosome, a microfilaria and a single unidentified protozoan, and their host distribution are recorded in Table 1.

Babesia wrighti. Three black-backed rock squirrels of the captured group were found to be naturally infected with a piroplasm which was subsequently reported as *Babesia wrighti*, n. sp., by the authors (1949). Blood films from five additional animals killed during the survey showed ring forms and rare young ameboid forms identical in appearance to those of *B. wrighti*. Since only one blood film from each of the five latter animals was available for study, the statement that these five were infected with *B. wrighti* is admittedly based on presumptive evidence only. Had we not had

TABLE 1

Host distribution of parasites

HOST SPECIES	NUMBER EXAMINED	POSITIVE FOR							TOTAL SHOWING ONE OR MORE SPECIES	
		Babesia wrighti		*Trypanosoma* sp.		Microfilaria		Unidentified Protozoa		
		Number	Per Cent	Number	Per Cent	Number	Per Cent	Number	Number	Per Cent
Fox squirrel (*Sciurus niger* ssp.)	140	4	2.9	18	12.9	23	16.4	1	39*	27.9
Gray squirrel (*Sciurus carolinensis* ssp.)	29					7	24.1		7	24.1
Black-backed Rock Squirrel (*Citellus variegatus buckleyi*)	75	4	5.3	9	12				12*	16
Say's Rock Squirrel (*Citellus variegatus grammurus*)	3									
Rio Grande Ground Squirrel (*Citellus mexicanus parvidens*)	28			2	7.1	2	7.1		3*	10.7
Prairie Dog (*Cynomys ludovicianus* ssp.)	37			2	5.4				2	5.4
Total	312	8+	2.6	31	9.9	32	10.3	1	63	20.2

* Includes double infections.

\+ Presumptive identification made from examination of single blood films in five animals.

the opportunity to study the course of the infection in the three live animals, positive identification of this protozoan would not have been possible. Indeed, the ring form of this organism so closely resembles that of *Plasmodium falciparum* that it was believed we were dealing with a plasmodium until detailed study of many slides over a period of three days revealed the presence of typical piriform bodies and the absence of pigment.

Trypanosoma sp. A trypanosome was seen in blood films from 31 animals. In four black-backed rock squirrels which were captured alive and found in the laboratory to be infected with trypanosomes, the parasite density was low, the animals appar-

ently suffered no ill effects from their infection, and after four months in captivity trypanosomes could no longer be found in thick blood films. In spite of the paucity of parasites, attempts were made to transmit this infection to the white rat and the white mouse by intraperitoneal injection of whole blood. These attempts were unsuccessful.

In addition to the thick blood films from trypanosome-infected animals, six thin films were available for study of parasite morphology. The organism varied considerably in size, the distinctly curved body measuring from 6.5 to 16μ in length and from 1.6 to 2.7μ in width with flagellum 6.4 to 10.7μ long. The relatively large, oval, deeply staining nucleus was always situated anterior to the central point of the body, being usually well within the anterior third. The kinetoplast was quite large, usually round and often situated in the extreme posterior extremity, although occasionally that portion of the body posterior to it was considerably prolonged and sharply pointed. The undulating membrane was narrow and showed few convolutions. This trypanosome, although smaller than *T. lewisi*, appeared otherwise to resemble that species. This is in general agreement with the previously described trypanosomes from the Sciuridae (Wenyon, 1926).

Microfilariae. Blood films from 32 animals showed microfilariae. The gray squirrel showed an infection rate of 24 per cent, the highest shown by any host species for any parasite in this survey. All of these infected gray squirrels and two of the similarly infected fox squirrels were killed in Oklahoma while the remaining animals came from Texas. Since species identification by microfilaria alone would be of questionable value, if not impossible, no attempt at further identification was made.

Unidentified protozoa. A single thick blood film from one fox squirrel showed an unidentified, predominantly crescent-shaped organism, 10 to 15μ in length by 2μ in width, having pale-blue, homogeneous cytoplasm and a large, oval, coarsely granular, darkly staining nucleus.

Double infections were observed in nine animals. Both trypanosomes and microfilariae were found in blood films of five *Sciurus niger* ssp. and one *Citellus mexicanus parvidens* while trypanosomes accompanied an infection with *Babesia wrighti* in two *Sciurus niger* ssp. and one *Citellus variegatus buckleyi*.

SUMMARY

1. Blood films were examined from 312 members of the family Sciuridae from Southwestern Texas and Southeastern Oklahoma. Three genera and five species were represented.

2. Twenty per cent of these rodents showed one or more parasites which included (a) a piroplasm identified as a new species, *Babesia wrighti*, in 2.6 per cent of the animals examined; (b) a trypanosome in 9.9 per cent; (c) a microfilaria in 10.3 per cent; (d) an unidentified protozoan in one animal.

3. Double infections were observed in nine of the rodents examined.

4. A blood film from an animal infected with *Babesia wrighti* may show only ring forms which closely resemble those of a plasmodium.

ACKNOWLEDGMENT

We are indebted to Dr. C. R. Eskey, Medical Officer in Charge, and to Mr. John S. Adams, Survey Aide, U. S. Public Health Service Laboratory, San Francisco, California for making available to us the facilities of the Plague Survey Team during whose operation the blood films were made.

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INVESTIGATIONS ON THE MOSQUITO TRANSMISSION OF *PLASMODIUM ELONGATUM* HUFF, 1930[1]

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Plasmodium elongatum Huff, 1930, has had relatively little use as an experimental malarial parasite. Even though it is known to have a rather wide geographic distribution it appears to represent a fairly small proportion of the avian plasmodia causing natural infections. The low peripheral parasitemia coupled with the difficulty in the laboratory of producing adequate infections in any of our common species of culicine mosquitoes and the inability to effect mosquito transmission of this parasite have limited its usefulness for malaria research.

One of the earliest investigations on the suceptibility of various species of mosquitoes to *P. elongatum* was that of Huff (1927) who found three species of the genus *Culex* to be susceptible to this parasite. One of the two *C. salinarius*, all of five *C. restuans* and 12 out of 47 *C. pipiens* were shown to be positive for oocysts after feeding upon infected birds. No development occurred in six species of *Aedes* or in one specimen of *Anopheles punctipennis*. In later experiments Huff (1932) reported two additional species of mosquitoes as being susceptible to *P. elongatum*. Of 18 *C. tarsalis* fed on an infected bird, three were positive for oocysts and one of nine *Aedes triseriatus* fed became infected after a previous (1927) unsuccessful attempt. Reichenow (1932) obtained no infections with *P. elongatum* through feeding 59 specimens of *C. pipiens*, 30 of *A. aegypti*, 10 of *Anopheles maculatus* and seven of *Theobaldia annulata* upon infected birds, whereas Raffaele (1934), working with an Italian strain of the parasite, obtained a 100 per cent infection in *C. quinquefasciatus* and 30 per cent in *C. pipiens*. In a study of *P. elongatum* in the duck, a single attempt by Wolfson (1946) to infect *C. pipiens* was unsuccessful, although she noted that gametocytes comprised a large proportion of the parasites seen in the peripheral blood. Raffaele (1934) has reported transmission of an Italian strain of *P. elongatum* through *C. pipiens* and *C. quinquefasciatus*. According to Wolfson (1946), Dr. R. D. Manwell was able to transmit it to the canary by means of *C. pipiens*; however, a direct report of this work has not been found in the literature. Huff, (1927, 1932) did not record a successful transmission of *P. elongatum* with any of the species used. He concluded that those species which gave entirely negative results are of little consequence in the transmission of avian malaria in nature and probably very seldom become infected.

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It was the purpose of the present investigation to determine the susceptibility of various species of mosquitoes to *P. elongatum* in different avian hosts and to accomplish mosquito transmission of the parasite.

EXPERIMENTAL

I. *Susceptibility of mosquitoes to P. elongatum in various avian hosts.*

The three vertebrate hosts utilized for this investigation were the common duck (*Anas buschas domestica*), the canary (*Serinus canarius*) and the English sparrow (*Passer domesticus*). Although the canary has been used most extensively, Wolfson (1946) found the duck to be a satisfactory host for *P. elongatum*. The English sparrow is the type host for this parasite.

Eight species of mosquitoes were tested for their susceptibility to the parasite. *Culex pipiens, C. quinquefasciatus, A. aegypti, A. atropalpus* and *Anopheles quadrimaculatus* were obtained from insectary colonies maintained in this laboratory. *Culex*

TABLE 1

Susceptibility of mosquitoes to P. elongatum *in different avian hosts.*

SPECIES	CANARY			SPARROW			DUCK		
	No. Fed	No. Pos.	% Pos.	No. Fed.	No. Pos.	% Pos.	No. Fed.	No. Pos.	% Pos.
C. pipiens	723	11	1.5	72	1	1.4	398	21	5.2
C. quinquefasciatus	78	0	0				47	0	0
C. restuans	182	4	2.2						
A. aegypti	168	0	0	89	0	0	65	0	0
A. atropalpus							52	0	0
A. triseriatus	82	2	2.4				27	1	3.7
A. vexans	10	0	0						
A. quadrimaculatus	47	0	0	20	0	0	21	0	0

pipiens, C. restuans, A. triseriatus and *A. vexans* were collected in the vicinity of Baltimore as eggs, larvae or pupae and bred out in the laboratory.

A total of 43 lots of mosquitoes, which included 2,181 specimens of three genera and eight species, were fed upon infected canaries, sparrows and ducks. The results of mosquito dissections are shown in Table 1. Since it was not possible throughout most of these experiments to find a species of mosquito which was sufficiently susceptible to *P. elongatum* in the canary to be used as a control, controlled feedings could not be carried out. In later experiments, however, with the duck as the vertebrate host, *Culex pipiens* was used as a control for certain infectivity experiments. In view of the fact that considerable variation existed in the results from one host to the other, these will be discussed separately according to host.

Canary. Of 723 *C. pipiens* comprising thirteen lots fed upon infected canaries, 11 or 1.5 per cent became infected. Seven of these lots contained no infected mosquitoes whatever. No correlation appears to exist between the number of gametocytes per 10,000 red cells present in the host at the time of feeding and the percentage of

infection in the mosquito. Although the highest percentage of mosquitoes with positive stomachs occurred in lot no. 1, which had obtained a blood meal from a canary with 500 gametocytes per 10,000 red cells, only 3.3 per cent of the individuals in lot no. 31 became infected when fed on a bird with 800 gametocytes per 10,000 red cells. All of the remaining lots having susceptible mosquitoes were fed on canaries with fewer than 100 gametocytes per 10,000 red blood cells.

The number of oocysts per individual stomach did not in any instance exceed three in number nor did any of them reach the stage of sporozoite development. On the contrary, regardless of the length of the incubation period no further development of the oocysts was observed beyond the ninth day. Their average diameter upon dissection of the stomachs 14 to 21 days after infective feedings was 27.6 microns. Despite the fact that the majority of them appeared to have a greatly thickened wall as seen in Figure 1, the general appearance of these oocysts was unlike

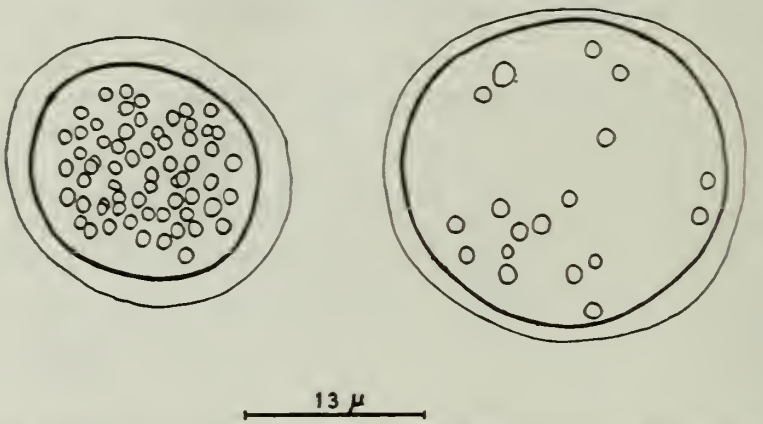

FIGURE 1. Typical oocysts of *P. elongatum* in *C. pipiens* mosquitoes infected from the canary showing their maximum size and development. Camera lucida drawings.

that of true, degenerate ones that have released their sporozoites. After reaching a certain stage of development they appeared to remain alive for two or three weeks but with no further growth, and a slow degenerative process ensued. Several lots of mosquitoes maintained at 24°C for as long as three weeks after feeding upon infected canaries failed to show a single stomach infection in which oocyst development had proceeded to maturity. The salivary glands of all mosquitoes with stomach infections were carefully examined and were found to be without sporozoites in every instance.

In order to determine whether or not a "wild" strain of *C. pipiens* would prove to be more susceptible to this particular parasite, lots no. 18 and no. 19 were reared from egg rafts and larvae secured from a temporary pool in Baltimore. Lot no. 18, composed of 96 specimens showed only a 2.4 per cent incidence of infection while all of the 70 mosquitoes in a second lot (no. 19) were refractory to the parasite.

Since Huff (1927) obtained a 100 per cent infection of *P. elongatum* in five *Culex restuans*, it was desirable to determine the infectivity of the present strain for this

species. A total of 182 specimens in lots no. 14, 15, and 16 were allowed to take a blood meal from two infected canaries with 50 to 100 gametocytes per 10,000 red cells at the time of feeding. Again there was no correlation between the percentage of gametocytes present and the incidence of infection in the mosquito. Mosquitoes of lot no. 15 fed on a canary with 50 gametocytes per 10,000 red blood cells showed the highest per cent of positives, 3, or 3.2 per cent; while the other lot (no. 16) with a single infection (2.5 per cent), had fed upon a canary with 100 gametocytes per 10,000 red cells.

In view of the negative results obtained by Huff (1927) in an attempt to determine whether *C. quinquefasciatus* was susceptible to *P. elongatum*, it seemed worthwhile to repeat this experiment with the present strain of parasite and mosquito. One large lot (no. 17) of *C. quinquefasciatus* (Alabama strain)[3] consisting of 78 specimens were fed upon a canary with 300 gametocytes per 10,000 red cells. All were negative for oocysts and sporozoites when dissected 14 days later. *Aedes triseriatus* was the only one of four species in this genus which proved to be susceptible to the parasite. Five lots of this species containing a total of 82 specimens took blood meals from canaries with from 85 to 260 gametocytes per 10,000 red blood cells. Although lot no. 4 showed a 14 per cent positive stomach infection, the total percentage of positives for all the lots was only 2.4. Even though a considerable number of mosquitoes was used in every experiment but one (*Aedes vexans*), no infections with *P. elongatum* were obtained in *C. quinquefasciatus*, *Aedes aegypti*, *Aedes vexans* and *Anopheles quadrimaculatus*.

Sparrow. Since the English sparrow is the original or type host of *P. elongatum* it was thought that a natural infection of the parasite in this host might prove to be more infectious for *C. pipiens* as well as other species. Only one of 54 laboratory bred *C. pipiens* became infected with the parasite (1.8 per cent) while 6.6 per cent of the specimens in a "wild" strain were susceptible when fed upon an infected sparrow. A series of five feeding experiments were set up using *A. aegypti* and *A. quadrimaculatus* in addition to *C. pipiens*. All mosquitoes in four of these lots failed entirely to become infected. A single specimen of *C. pipiens* in lot no. 20 showed one oocyst upon stomach dissection.

Duck. Although Wolfson (1946) failed in a single attempt to infect *C. pipiens* with *P. elongatum* in the duck it seemed worthwile to carry out a preliminary experiment with this host in order to determine its suitability for further infectivity and transmission experiments. A total of 78 C. pipiens in the first lot (no. 32) took a blood meal from a young duck showing 1000 gametocytes per 10,000 red blood cells. These were maintained at room temperature (23°C) for 13 days prior to dissection, at which time five specimens contained oocysts. All stomachs contained a single cyst, except one with five oocysts, and all were mature, but the 19 days which had elapsed since taking the blood meal was apparently not a sufficient length of time to allow the sporozoites to reach the salivary glands since they were found to be negative. Inasmuch as the resulting infection in this lot of mosquitoes was considerably higher than any obtained from canaries infected with *P. elongatum*, it seemed desirable to concentrate on the duck as the vertebrate host for all subsequent infectivity and transmission

[3] Referred to by Farid (1948) as a hybrid strain.

experiments. *C. pipiens* was always used as a control in feeding experiments involving species of mosquitoes which were not known to be susceptible to *P. elongatum.* Prior to conducting such an experiment the stomach contents of a recently fed *C. pipiens* were examined for exflagellation in order to assure a prevalence of numerous mature gametocytes of both sexes.

Most of the mosquitoes other than *C. pipiens* did not become infected by feeding on the duck. Lot no. 38 was composed of a number of *A. quadrimaculatus, A. aegypti* and *C. pipiens* in order to ascertain the infectiveness of *P. elongatum* in the duck for the first two species with the latter used as a control. The only infection was found in a single *C. pipiens* of the 15 dissected. Of 47 *C. quinquefasciatus* which had fed on a duck showing 1100 gametocytes per 10,000 red blood cells after the demonstration that exflagellation was occurring in the stomachs, all were negative upon dissection. A total of 65 *A. aegypti* failed to become infected after feeding on a bird with a considerable number of gametocytes. All of 21 *A. quadrimaculatus* likewise were insusceptible to the parasite. Again, the only culicine species other than *C. pipiens* which proved to be susceptible to *P. elongatum* was *A. triseriatus,* but only one of 27 fed became infected.

Table 1 shows that the incidence of infection in *C. pipiens* is somewhat higher after the mosquitoes had fed upon infected ducks than it was following infective meals on canaries or sparrows. Furthermore, oocyst development of *P. elongatum* in *C. pipiens* which had received their infectious blood meal from the duck was quite unlike that occurring in the mosquito when fed upon infected canaries. In the former the oocysts generally completed their development in 10 to 12 days. Mechanical rupture of such cysts generally demonstrated a slight motility on the part of the sporozoites. In mosquitoes with comparatively light stomach infections (less than 10 oocysts), all oocysts showed approximately the same degree of development. When their numbers were in excess of this, a considerable difference in the developmental stage was noted between them. Figure 2 demonstrates these differences as they occurred on the same stomach. Although all drawings were made on the same day there is a marked variation in size of the oocysts irrespective of the stage of development and likewise, there is a similar variation in size between oocysts showing the same stage of growth. Whereas Figures 2A and 2B represent immature oocysts in the early pigment granule stage, one is more than double the size of the other. Similarly, Figures 2E, 2F and 2G demonstrate the marked size variation which occurs in spite of the fact that all three represent fully mature oocysts full of sporozoites. Figures 2H and 2I show degenerate oocysts which have released their sporozoites.

II. *Selection of C. pipiens for susceptibility to P. elongatum.*

Preliminary experiments designed to increase the susceptibility of *C. pipiens* to *P. elongatum* from the canary were unsuccessful due to the extremely low infection rate in the mosquito when fed upon this particular avian host. When it became apparent that this species of mosquito was considerably more susceptible to the same parasite in the duck, it was possible to carry out a series of selection experiments. The purpose of these studies was to determine whether susceptibility of a strain of *C. pipiens* to *P. elongatum* could be increased through the selection of progeny from

infected females. If such an experiment did prove to be successful, it was then planned to use the more susceptible strain in attempts to transmit the parasite through the mosquito to the duck.

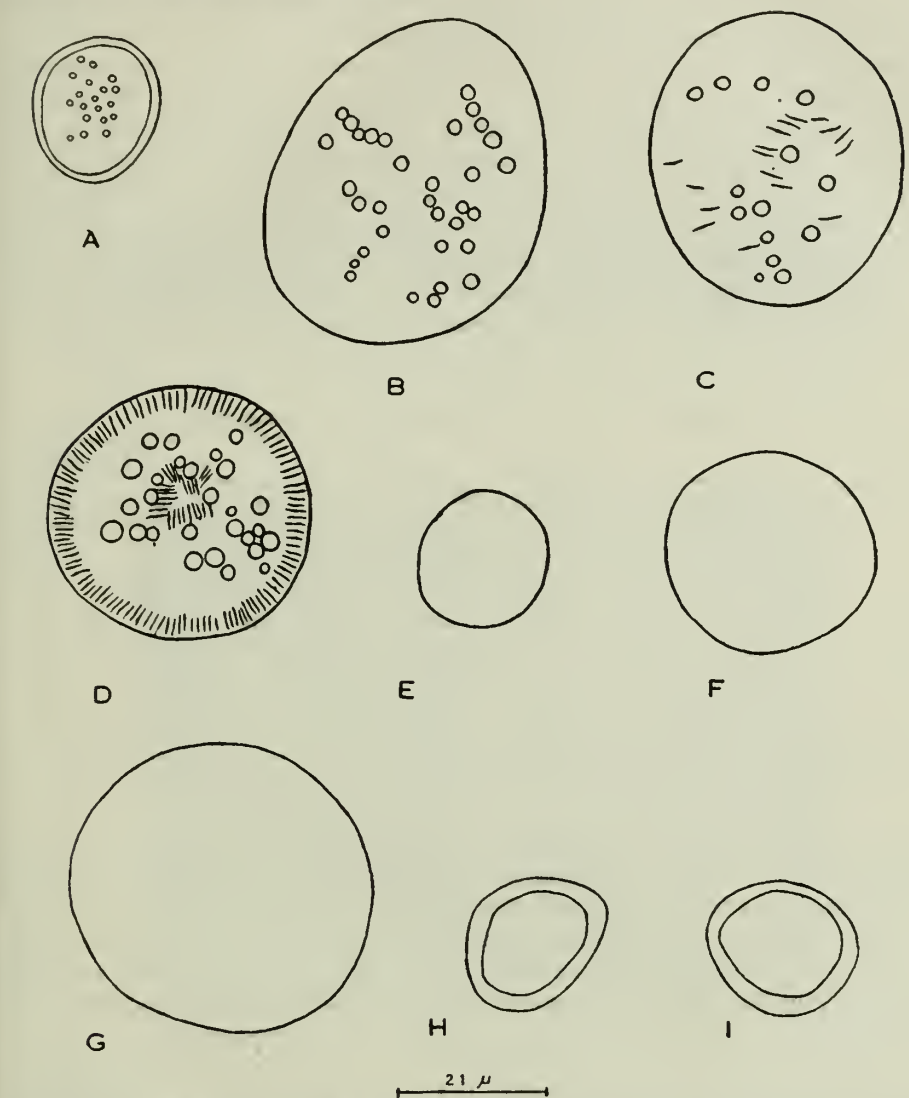

FIGURE 2. Oocysts of *P. elongatum* from a single *C. pipiens* infected from the duck. Note the marked variation in development occurring in oocysts of the same age. Camera lucida drawings.

The procedure, with certain modifications, was essentially that used by Huff (1929). A group of mosquitoes were allowed to feed on a suitable duck, and the engorged ones were removed to another cage. After seven days these females were placed in individual test tubes, half full of water. A small piece of cheesecloth held in place by a rubber band covered the opening. After deposition of the ova or after

the mosquito died, it was dissected. If oocysts were present the egg raft from the infected female was placed in water in a rearing pan and the progeny raised to adulthood. For each generation so fed a corresponding control, consisting of a lot of *C. pipiens* from the regular colony which received a blood meal from the same duck on the same day, was set up. The results of these experiments are shown in Table 2. Although generation I showed a smaller percentage of infected individuals than the corresponding control group a gradual increase in susceptibility of the selected strain was noted, beginning with generation II. The fourth generation was divided into two groups on the basis of the number of oocysts present on the stomachs of each of two females of the preceding generation. One set of generation IV progeny came from a female with 3 oocysts and the other set from a female with 12 oocysts. Generation IVa, derived from the 3-oocyst female showed a lower percentage (21.0) of positive individuals than the generation IVb which had an infection rate of 35 per cent. The control group fed on the same duck showed only a 6 per cent infection.

TABLE 2

Selection of C. pipiens *for its susceptibility to* P. elongatum

GENERATION	DERIVED FROM — ♀ ♀ with — oocysts	GAMETOCYTES/ 10,000 RBC	NO. DISSECTED	NO. POSITIVE	PERCENT POSITIVE	CONTROL	
						No. Dissected	Percent Positive
I	—	500	38	5	13	46	15
II	1:8	1400	35	6	17	32	9
III	2:3, 9	1000	41	8	19.5	37	11
IVa	1:3	1200	14	3	21	24	6
IVb	1:12	1200	17	6	35	24	6
V*	3:9, 14, 22						
VI	All progeny pooled	1400	41	20	48.8	49	13

* This generation was fed upon an uninfected duck.

Due to certain technical difficulties, an infected duck was not available for feeding fifth generation females. Consequently, it was necessary to allow them to secure a blood meal from a clean (uninfected) duck in order to maintain the susceptible strain. Egg rafts from the eleven survivors were pooled and reared through as usual. Of 41 generation VI females which fed upon a duck showing 1400 gametocytes per 10,000 red blood cells, 20 or 49 per cent became infected. In the corresponding control group only 13 per cent of the females proved to be susceptible to the parasite.

Figures 3A and 3B indicate the density of oocyst infection that may be reached in the more highly susceptible individuals by the sixth generation. It will also be noted in Figure 3 that the oocysts are more uniform in size and development than those shown in Figure 2.

III. *Transmission of P. elongatum through the mosquito.*

It has been shown above that *P. elongatum* from the canary did not progress beyond the early oocyst stage on the stomach of *C. pipiens*; but that this parasite was

able to complete its development through the sporozoite stage in the same species of mosquito when the latter received its blood meal from an infected duck. It was the purpose of this experiment to attempt to bring about an infection in a clean duck by means of the sporozoites.

Previous dissections had shown that sporozoites were present in the salivary glands in relatively small numbers. It was therefore decided that large numbers of

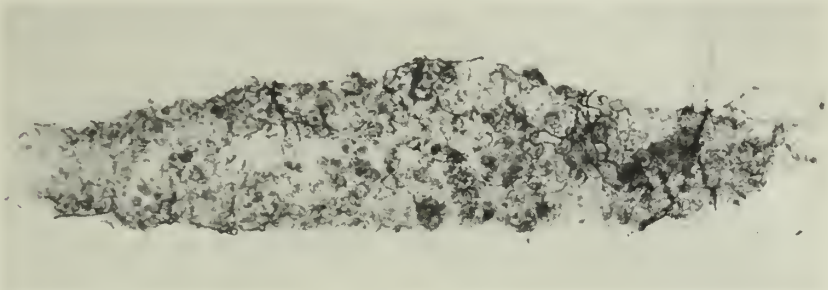

FIGURE 3A. The stomach of a sixth-generation selectively-bred *C. pipiens* female showing large number of oocysts of *P. elongatum*. (× 60).

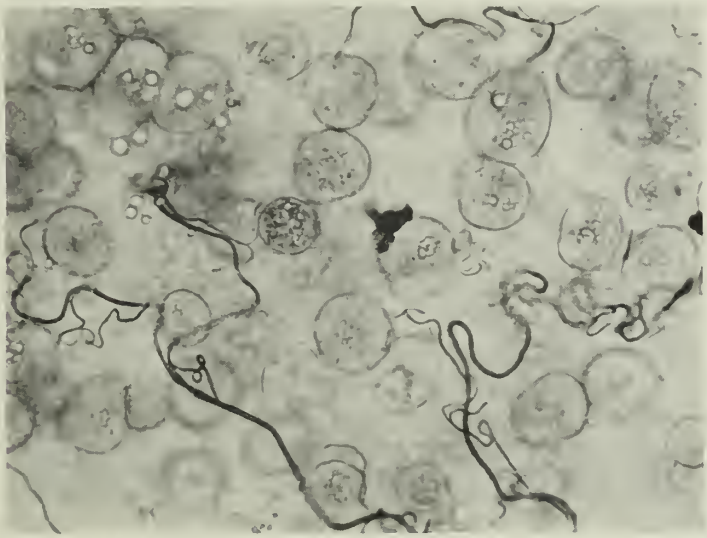

FIGURE 3B. A portion of the stomach seen in Figure 3A. Both mature oocysts and those which have released their sporozoites may be seen. (× 300).

mosquitoes should be employed in any attempt to transmit the parasite from duck to duck. In the first transmission experiment 336 *C. pipiens* mosquitoes received a blood meal from a duck exhibiting 100 gametocytes per 10,000 red blood cells. The mosquitoes were maintained at a constant temperature of 27°C and relative humidity of 85 per cent for a period of fourteen days. Dissection of eleven specimens prior to this time had revealed sporozoites in the glands on the thirteenth day after

the infective blood meal. The entire lot of mosquitoes was then given the opportunity to feed upon a clean duck. Approximately 127 of them were induced to take this second blood meal. Daily blood smears were made on the clean duck and parasites were first noted on the twelfth day following the mosquito feeding. Fewer than 10 parasites per 1000 red blood cells were present on this first day of the infection. When it became evident that the degree of parasitemia was not going to increase, this mosquito-transmitted strain of *P. elongatum* was subinoculated to a clean duck and studied daily throughout the period of patent parasitemia. There was a gradual rise in the number of parasites per 1000 red cells to a peak of 17 on the ninth day following inoculation. In an effort to determine whether any genetical change had been produced in the strain through mosquito transmission it was carefully compared with the blood-induced strain of *P. elongatum* in the duck through the next six passages of both. One hundred parasites were counted at the peak of the infection after each transfer and the percentage of gametocytes compared with that of the asexual stages (schizonts and trophozoites). With the exception of one passage, the percentage of gametocytes was consistently lower in the mosquito-transmitted strain than in the regular, blood-induced one. Numerous additional transfers must be made, however, before one could conclude that this modification in the strain after passage through the mosquito is of a permanent nature. No morphological differences between the gametocytes or other growth stages of the two strains were apparent. Likewise the percentage of multiply-infected cells and the ratio of microgametocytes to macrogametocytes were essentially the same. Lack of time precluded further experiments to determine any variation in the infectiousness of the mosquito-transmitted strain for mosquitoes or in its transmissibility through the latter to canaries as well as ducks.

DISCUSSION

Considerable variation exists between the present findings and those of other investigators as to the susceptibility of various mosquito species to *P. elongatum*. Although the same species found to be susceptible to the parasite by Huff (1927, 1932) were likewise susceptible here, the infection rates for these species were considerably lower than those obtained by him. This may, however, be partly due to the fact that relatively small numbers of mosquitoes such as those employed in his experiments give highly erratic percentages. In his earlier studies (1927), for example, Huff obtained a 26 per cent infection rate in 47 *C. pipiens*. When the number of mosquitoes fed on infected birds was increased to 109 (1932) the percentage of infectivity was 13 per cent. The fact that Reichenow (1932) was unable to infect this species with *P. elongatum* while Raffaele (1934) found 30 per cent of the *C. pipiens* susceptible indicates a physiological variation in either the strains of parasites, or mosquitoes, or perhaps both. As pointed out by Huff (1938) each malarial parasite, mosquito and vertebrate host differs genetically from its parents. Consequently, it seems most likely that these three components are constantly undergoing genetic change, and, in addition, further alterations are brought about by such factors as isolation and selection. This is further demonstrated by the fact that both Huff

(1927) and the writer were unable to infect *C. quinquefasciatus* with the parasite although Raffaele (1934) reported 100 per cent successful results. A much lower degree of infection of *P. elongatum* in *C. restuans* was obtained here than by Huff (1927, 1932). It must be noted again, however, that the 100 per cent infection rate which he produced in this species was based upon a total of five mosquitoes. The low degree of susceptibility of *A. triseriatus* in the latter study compares with the present results. Since three of the studies, including the present one, demonstrate completely negative results with *A. aegypti*, it seems fairly certain that this particular species is not a vector of *P. elongatum* in nature.

During attempts to infect *C. pipiens* mosquitoes with *P. elongatum* from the canary it became evident that the parasite had lost much of its infectiousness for the mosquito. It appeared that some factor or factors were not only suppressing ookinete penetration of the stomach wall but were also preventing complete development of the oocysts. That such an inimical factor was not manifest prior to this stage was demonstrated by the fact that the gametocytes of *P. elongatum* from the canary were able to exflagellate and fertilize in the usual fashion, in the stomachs of *C. pipiens* mosquitoes. Since there was no correlation between the relative numbers of gametocytes and the numbers of mosquitoes positive for infection, the lack of susceptibility of *C. pipiens* to *P. elongatum* cannot be explained upon the basis of inadequate numbers of gametocytes in the circulating blood of the host. Several of the more highly infected lots received their blood meals from birds showing the smallest number of gametocytes. The fact that a "wild" strain of *C. pipiens* was no more susceptible to *P. elongatum* than the colony strain may indicate that a gradual decrease in infectiousness for the mosquito has occurred in the parasite, rather than an increased resistance on the part of the laboratory-reared *C. pipiens*. It is of interest to note here that when a number of wild-caught *C. pipiens* were fed upon a sparrow with a natural, low-grade infection with *P. elongatum*, the single specimen with a stomach infection showed a mature oocyst. Thus, there is some further indication that the regular laboratory strain of the parasite had lost some of its infectiousness for the mosquito. The reason for the complete insusceptibility of both *A. aegypti* and *A. quadrimaculatus* to *P. elongatum* was not discovered until subsequent studies, (Micks, DeCaires and Franco, 1948) were made. The parasite was found to be incapable of completing exflagellation in the stomachs of either species.

It seemed of unusual interest that when *C. pipiens* from the same laboratory colony were allowed to feed upon ducks (abnormal hosts) with the same strain of *P. elongatum*, they exhibited a much higher degree of susceptibility not only in the infection rate but also in the degree of oocyst development which in this case was always complete. It appears that some factor is present in a particular fraction of the duck blood which either causes the gametocytes to be more resistant to any adverse environmental conditions encountered in the stomach of the mosquito or somehow increases the infectiousness of the gametocytes. Such a factor seems to be lacking in canary blood. It is the opinion of the writer that feeding several lots of mosquitoes on infected canaries both before and after transfusion of various blood fractions from the duck might throw further light on the problem. Using this method,

Cantrell and Jordan (1946) obtained some indication that depletion of an essential nutrient in the blood of the host as a result of the high parasitemia had in turn lowered the threshold of infectability of gametocytes for the mosquito.

The findings presented in Table 2 indicate that heredity is a factor in determining the susceptibility of *C. pipiens* to *P. elongatum* (in the duck). Selective breeding from infected females through six generations resulted in an increase in susceptibility of from 13 per cent to almost 50 per cent. Infection in the control mosquitoes never exceeded 15 per cent and in one case only 6 per cent were susceptible. That such characters as these are not always inherited is demonstrated by the work of Jeffery (1944) and Hovanitz (1947) in which selective breeding brought about no increased susceptibility on the part of the mosquitoes used. The findings of Huff (1929, 1931) and Trager (1942), however, were somewhat similar to those of the present study. An interesting phenomenon observed in this experiment was that although fifth generation females were fed upon an uninfected duck, a marked rise in the incidence of infection occurred in the following generation. While 35 per cent of the *C. pipiens* of generation IVb were infected, about 49 per cent of those in the sixth generation were susceptible to the parasite. The fact that the number of gametocytes per 10,000 red cells was somewhat higher in the sixth generation than in the fourth may in part explain these results. Since the progeny (generation IVa) from a female with 3 oocysts showed a considerably lower incidence of infection (21 per cent) than those (generation IVb) from a female with 12 oocysts, it is possible that a more highly infected female mosquito will produce more progeny which will be susceptible to the same parasite than a mosquito with an extremely light stomach infection.

The successful transmission of *P. elongatum* from duck to duck through the mosquito is rather surprising inasmuch as it has not to date been found in nature to be a parasite of any of the group of *anseriform* birds to which the common duck belongs. It seems quite possible, however, that large numbers of sporozoites are necessary in order to effect transmission and that under natural conditions transmission of this parasite to the duck may only occur rarely if at all. It is not possible at present to explain why the abnormal host furnished gametocytes which could develop to infectious sporozoites, while the canary did not. It might be assumed that a mutation had occurred; nevertheless transmission of *P. elongatum* from the duck through *C. pipiens* does not appear to have produced any drastic alteration in the strain. It has been noted, however, that the number of gametocytes has been appreciably reduced with a corresponding rise in sexual stages. Other alterations may have occurred which will not become apparent until the strain is inoculated into the canary or until attempts have been made to transmit it through the mosquito to the canary. Huff (1941) and Redmond (1943) both noted very definite and apparently permanent alterations in strains of avian malaria after passage through the mosquito.

The long prepatent period of 11 days in the duck receiving its infection from the mosquito (*C. pipiens*) may partly be explained by the fact that the salivary gland infections produced by *P. elongatum* in this mosquito were comparatively light. The number of sporozoites introduced into a host is known to have a direct

effect upon the length of the prepatent period. Another explanation for the long prepatent period and the low-grade patent parasitemia may be that the parasite has lost some of its pathogenicity for the duck as a result of mosquito passage.

SUMMARY AND CONCLUSIONS

Culex pipiens, *C. restuans* and *A. triseriatus* were found to be susceptible to *P. elongatum* from the canary, only to the point of partial oocyst development. It was not possible to infect *C. quinquefasciatus*, *Aedes aegypti*, *A. vexans* or *Anopheles quadrimaculatus* with this parasite from the same host. A "wild" strain of *C. pipiens* was susceptible to *P. elongatum* from both the canary and the English sparrow. *Culex pipiens* and *A. triseriatus* were both infected with this parasite in the duck. *P. elongatum* was able to complete its entire development through the sporozoite stage in the former species of mosquito. Salivary gland infections, however, were comparatively light. *Culex quinquefasciatus*, *Aedes aegypti*, *A. atropalpus* and *Anopheles quadrimaculatus* were insusceptible to stomach infection by *P. elongatum* in the duck.

Through selective breeding from females exhibiting stomach infections with *P. elongatum*, it was possible to increase the susceptibility of *C. pipiens* to this parasite from 13 per cent to approximately 49 per cent within six generations. There was some indication that those females with the greatest number of oocysts per stomach gave rise to the largest number of susceptible progeny.

It was possible to transmit *P. elongatum* from duck to duck through *Culex pipiens*. The prepatent period was 12 days and the resulting infection was of a very low grade. In seven subsequent blood transfers of this strain from duck to duck, the percentage of gametocytes in this mosquito-transmitted strain was consistently lower than in the regular, blood-induced one. Whether any definite changes of a genetical character resulted from this mosquito passage of the parasite is not known.

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PARASITES RESEMBLING *PLASMODIUM OVALE* IN STRAINS OF *PLASMODIUM VIVAX*

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During a study of military patients with malaria and during the treatment of paresis with *Plasmodium vivax*, there were observed malaria parasites with characteristics similar to those described for *Plasmodium ovale*. These abnormal forms were seen in the Chesson and Pait strains of *vivax* from New Guinea and in the St. Elizabeth's strain from the United States.

The Chesson strain was isolated in 1944 by this laboratory (Ehrman, Ellis, and Young, 1945) and has been used continuously since then in the treatment of paresis and in experiments with new synthetic drugs.

The Pait strain was isolated from a Marine relapsing with malaria in January of 1945 and was maintained by this laboratory until February, 1947. The original transfer to mental patients was made because of the observation of a few mature segmenters with 10 or less merozoites.

The history of the St. Elizabeth's strain has been previously recorded (Coatney and Young, 1941).

METHODS

Most of the observations reported were made from blood smears taken routinely each day on neurosyphilitic patients undergoing malaria therapy. The stain used was Giemsa and morphological studies were made from thin films.

OBSERVATIONS

Morphology of the segmenter. The segmenter is one of the most diagnostic stages of *P. ovale*. The infected erythrocyte is usually enlarged, stippled, and blanched. The parasite is about the size of *P. malariae* and is said to have from 6 to 14 merozoites, averaging about 8.

In several instances we saw segmenters of the St. Elizabeth's strain of *P. vivax* with as few as seven merozoites.

In the Pait strain of *P. vivax*, segmenters with as few as four merozoites have been seen. Considerable variation in merozoite numbers occurred on one occasion; 76 segmenters exhibited from 4 to 11 merozoites, averaging 6.3. This is shown as a frequency graph in figure 1 (upper graph).

Also, the Chesson strain of *P. vivax* would occasionally show segmenters with a reduced number of merozoites. In one instance, 351 segmenters showed a range of

[1] Grateful acknowledgment is made to Miss Inez Demonet, National Institutes of Health, who kindly prepared the colored drawings.

merozoites from 6 to 21, averaging 11.7 (figure 1, lower graph). About 10 per cent of the segmenters had 16 or more merozoites.

In both of the cases shown in figure 1, the frequency curve is unimodal with some

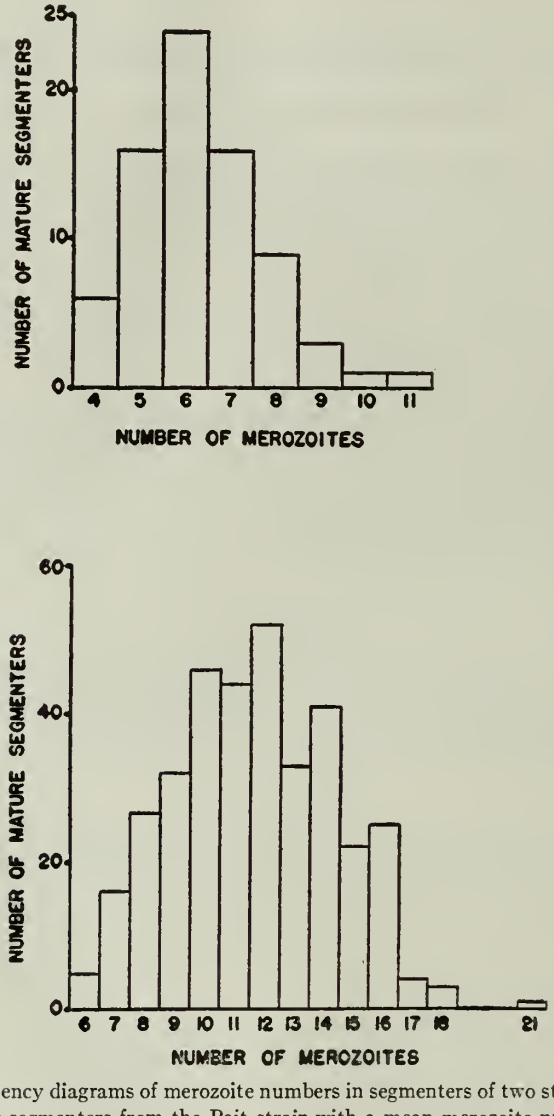

FIGURE 1. Frequency diagrams of merozoite numbers in segmenters of two strains of *Plasmodium vivax*. Upper graph: segmenters from the Pait strain with a mean merozoite number of 6.3. Lower graph: Chesson strain segmenters with a mean merozoite number of 11.7.

positive skewness. In other cases where a sufficient number of segmenters were counted to justify plotting a curve, a similarly-shaped curve was found.

The size of these abnormal segmenters varied with the merozoite number except in the cases with exceptionally small numbers in which the individual merozoites

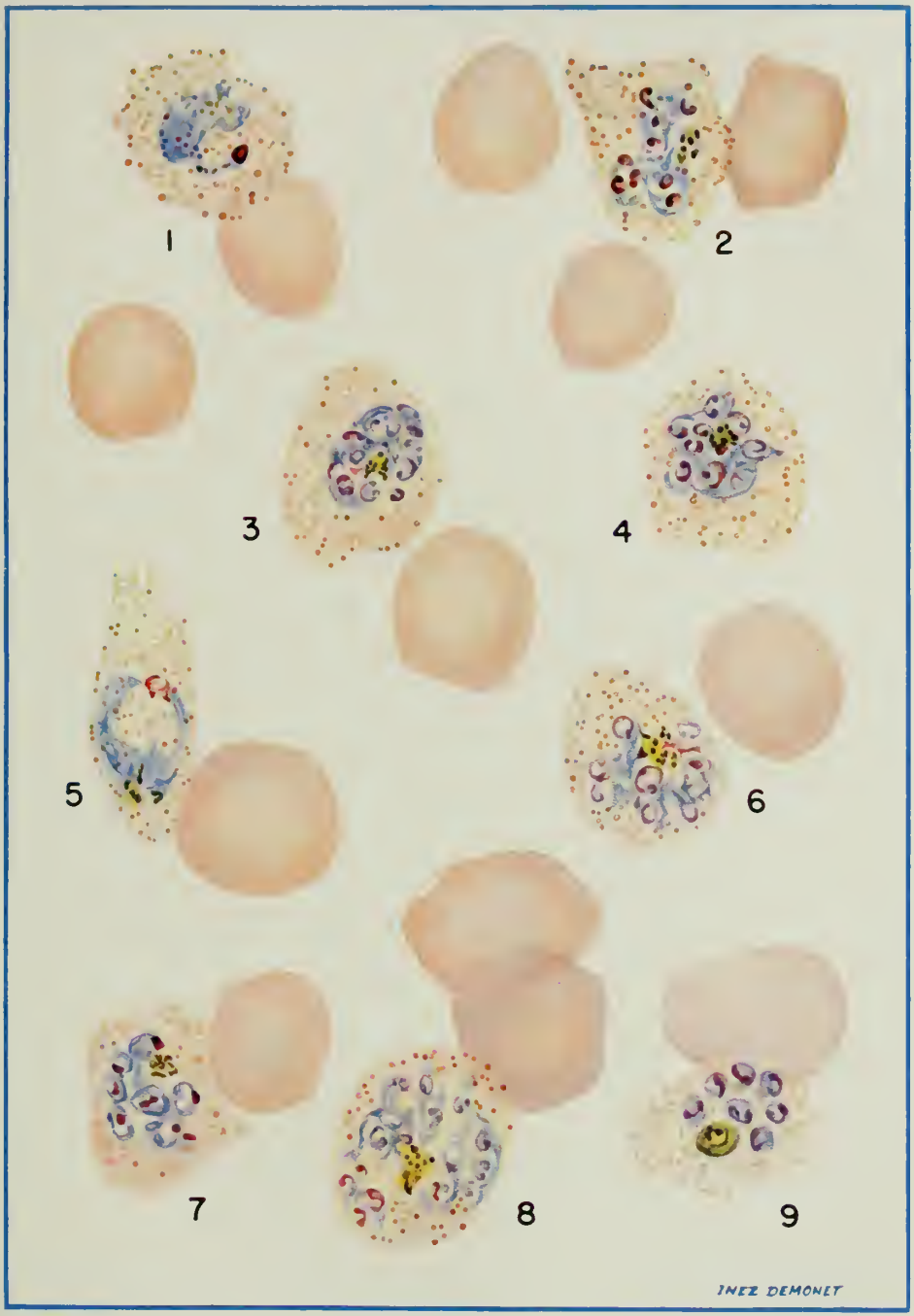

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PLATE 1. Parasites resembling *P. ovale* in strains of *P. vivax*.
Figs. 1–8, Chesson strain. Fig. 9, Pait strain.

Fig 1. Immature form showing sluggish appearance of parasite. The red blood cell has fimbriated edges.

Fig. 5. Immature parasite in red blood cell, the latter showing oblong shape with fimbriation.

Figs. 2, 3, 4, 6, and 7. Segmenters with a small number of merozoites.

Fig. 8. A segmenter more typical of *P. vivax* which occurred along with the others.

Fig. 9. A small segmenter in an *ovale*-shaped red blood cell.

were larger than in normal *vivax*. The entire segmenter occupied from one-half to two-thirds of the enlarged red cell. Usually the differentiation of the merozoites, particularly the nuclear elements, was poorer than in the case of normal *P. vivax*.

The red cells were enlarged, blanched, and stippling with Schuffner's dots was heavy. The pigment was usually abundant, heavily clumped, and centrally located, often giving a rosette-like appearance to the segmenter.

Plate 1 illustrates segmenters and other parasites from the Chesson and Pait strains. Figures 2, 3, 4, 6, and 7 of this plate show *ovale*-like segmenters from the Chesson strain with merozoite numbers varying from 6 to 10. Figure 8 illustrates a segmenter typical of *P. vivax* which occurred along with the others. Figure 9 shows a small segmenter from the Pait strain.

Morphology of the trophozoite and gametocyte. Most of the parasites other than the segmenters conformed well with those ordinarily seen in *vivax* except for the smaller size. Trophozoites which stained heavily and showed little tendency for pseudopodia formation were seen (plate 1, figures 1 and 5). A small number of parasitized erythrocytes, however, showed oval shapes and fimbriated ends. The gametocytes were essentially similar to those ordinarily seen in *P. vivax*, except often smaller.

Multiple-infected red blood cells. In many of the cases with abnormal segmenters the number of multiple-infected red cells was much higher than that usually found in *P. vivax*. Frequently 10 to 20 per cent of the newly parasitized red cells were multiply infected and cells with four, five, six, and even seven rings were not uncommon. It might be pointed out that the parasite density concurrently observed was very high ranging up to 50,000 per cubic millimeter or above. Whether these frequent multiple infections of a single cell were due to the high parasitemia or to the invasion of cells by parasites containing plural nuclear complements cannot be answered at this time, the latter hypothesis being one which would require much more proof than we have at the present.

Time of appearance in patients. The studies on the abnormal segmenters are based primarily upon routine smears taken daily on the mental patients; consequently, since the segmentation time of the parasite and the time of making the smear only occasionally coincided, the abnormal forms might not have been detected in many instances.

As was stated in the introduction, the Pait strain was originally put into mental patients because of the finding in a Marine of a few segmenters with a reduced number of merozoites. Since then the strain has been observed through more than six serial passages and abnormal segmenters have been seen in 9 of 18 patients.

In the Chesson strain such abnormal segmenters have been less frequently observed. Only 2 of over 200 patients have been noted to have the forms and we have seen no record of such forms being observed by other laboratories working with this strain.

P. ovale-like segmenters have been seen upon only two or three occasions in the St. Elizabeth's strain which has been used continuously for over 10 years in the treatment of neurosyphilis.

The fact that routine daily smears were used in this study also prevents us from stating with certainty the facts regarding the prevalence of these forms at the various

stages in the course of the disease. It can be stated that *ovale*-like segmenters were found from the sixth to the fourteenth day after the first fever. Two cases showed many abnormal segmenters from the sixth to the tenth day after the first fever, which corresponds to the period during which maximum parasitemia was reached. One patient who had the *ovale*-like forms very abundantly from the sixth to eighth day after first fever was found to have many normal segmenters and only few *ovale*-like forms from the eleventh to the fourteenth day. All of the patients showed normal *vivax* segmenters either concurrently with the abnormal or during some other stage of the disease.

DISCUSSION

The parasites we have observed showed two main points of resemblance to *P. ovale:* 1. Some segmenters had small numbers of merozoites, occasionally fewer than eight. On some slides only segmenters with the reduced number of merozoites were seen. 2. Some of the infected red blood cells were oval-shaped, some were fimbriated, and some were very heavily studded with Shuffner's dots.

In contrast, several features were observed which are incompatible with a diagnosis of *P. ovale:* 1. The infections often caused rigorous courses in the patients; whereas, *P. ovale* is ordinarily quite mild and prone to spontaneous cure. 2. The infections frequently relapsed; whereas, *P. ovale* is supposed to relapse only infrequently. 3. *Ovale*-like parasites did not occur continuously throughout the course of the disease in the patients. *Vivax*-type parasites were invariably seen in the same patients showing the *ovale*-like forms, although in some instances the two forms did not occur simultaneously. 4. In our series of transfers, many patients showed none of the abnormal forms. Even in the Pait strain such parasites were detected in only one-half of the patients.

The possibility of these infections (especially the Pait strain) being a mixed infection of *P. ovale* and *P. vivax* must be considered, and the following points would indicate such is not the case: 1. The unimodal frequency distribution of the number of merozoites in the segmenters would suggest a single species. Had both species been present one would expect a bimodal curve with one peak near 8 merozoites and the other near 16. 2. The *ovale*-like parasites did not appear consistently in consecutive transfers.

The above experience indicates that parasites resembling *P. ovale* can occur in established strains of *P. vivax.* Furthermore, such abnormal forms may occasionally be the prevailing type. Had not our original findings in the Pait strain been followed up with continued observations on the patient and by sub-inoculations into mental patients, it is likely that an erroneous diagnosis of *P. ovale* would have been made.

Giovannola (1935) argues that *P. ovale* is a modification of *P. vivax* in chronic infections and after long continued passage by blood from one person to another. He thought that not enough proof was present to accept *P. ovale* as a fourth human *Plasmodium.*

Our findings do not necessarily support Giovannola's contentions. The conditions under which we found these abnormal forms are not the same as those described by Giovannola. The infrequency with which we found the segmenters with small num-

bers of merozoites indicates a transient host-parasite relationship rather than a permanent one. As these forms were usually found about the time of maximum parasitemia, it seems that the high parasite density might be correlated with the small numbers of merozoites. A similar association was described by Boyd (1939), who, working with *P. cathemerium*, found in some birds that the merozoite number dropped from 16 on the first day of infection to 8 on the fourth day, the latter being the day of maximum parasitemia. As the number of parasites decreased subsequently, the number of merozoites increased although not reaching the initial level.

The observations made in this study do not necessarily indicate that *P. ovale* is not a good species. The experimental work of James, Nicol, and Shute (1932, 1933, and 1935) indicates that it is a valid species. However, it is believed that many of the reports of *P. ovale*, especially outside of Africa, involving isolated cases where the diagnosis is made upon a single or only a few slides, are subject to question as they may be based upon aberrant forms of *P. vivax* such as we have observed.

SUMMARY AND CONCLUSIONS

Parasites resembling *Plasmodium ovale* have been seen in two strains of *Plasmodium vivax* from New Guinea and in one strain from the United States. Such parasites appeared for only short periods of time in any one patient and were demonstrated only infrequently in patients receiving the malaria in serial transfers. The segmenters showing a reduced number of merozoites seem to be related to the parasite density, as they occurred about the time of maximum parasitemia.

It is believed that the *ovale*-like forms were aberrant parasites of *P. vivax*.

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A WINTER STUDY OF *ANOPHELES* MOSQUITOES IN SOUTHWESTERN GEORGIA, WITH NOTES ON SOME CULICINE SPECIES

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The present study was undertaken to extend previous observations on the overwintering activities of *Anopheles quadrimaculatus* Say in a malarious section of southwestern Georgia. Similar study of the other common *Anopheles* in the region, *punctipennis* (Say) and *crucians* Wied., and of culicine mosquitoes was to be undertaken collaterally as opportunities might occur.

Some twenty-odd years ago, Barber, *et. al.* (1924) suggested that effective malaria control operations directed against the mosquito vector of the disease might be executed in winter. Success from such a program in the southeastern United States presupposes a thorough knowledge of winter biology of *Anopheles quadrimaculatus*, but the overwintering habits of *A. quadrimaculatus* are not adequately known. In 1940 Hinman and Hurlbut summarized the previous winter studies of this species. They conclude that true hibernation by *A. quadrimaculatus* does not occur in the southern United States and that spring populations of this species originate from overwintering females supplemented in some areas, perhaps, (as believed by Barber, *et. al.* (1924)) by development of retarded larvae.

PROCEDURES

Most of the observations and collections on which this study is based were made between December 1, 1944 and March 31, 1945 in the western part of Baker County, Georgia. A few observations were made in the immediately adjacent eastern part of Early County, Georgia. The observations were in part repeated during the corresponding period in the winter 1945–46.

Throughout the study, searches were made for larval and adult mosquitoes at various intervals in each of the winter months. The localities of three aquatic situations, Mossy Pond, Springfield Pond, and DeSoto Spring, were visited often (once every seven to ten days), and provided the majority of the specimens although occasional collections were made at ten other ponds in the study area.

Larvae were collected by sweeping surface floatage with a shallow rectangular enamelware pan. Each sweeping dip sampled approximately two square feet of water surface, and at each collection 50 or more dips were made. (Surface sampling was supplemented by stirring the water to recover larvae which might be resting on the bottom, but in no instance were larvae obtained by this procedure). Larvae and pupae were pipetted from pan to collection bottles and were identified by microscopical

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examination at the laboratory. All identifications were based upon examination of fourth instar larvae or reared adults.[2]

Adult specimens were collected directly from artificial resting places or were obtained by fumigation of hollow trees. The artificial resting places were 39 wooden boxes: 32 near the margin of Mossy Pond and 7 at the edge of Springfield Pond. The wooden boxes, as described by Goodwin (1942) were painted red on interior and exterior surfaces, were one foot in cubical dimension, had one vertical face open, and were nailed to trees or other supports approximately five feet above ground level. In addition to the routine collections from red boxes, 47 buildings on three farms in the area were examined for adult mosquitoes in March, 1945, and 80 buildings were examined in late March, 1946.

Hollow trees were fumigated with sulfur dioxide gas generated by burning a mixture of sulfur and fuel oil in an improvised burner. The burner was a cylinder of hardware cloth suspended in a larger cylinder of tin, the outer cylinder being held upright by a wire attached to its side. For the fumigation of a tree approximately 75 grams of sulfur were mixed into a thick paste with fuel oil, and the mixture spread on a piece of cheese cloth. The cheese cloth was then wrapped around the inner cylinder of the burner and ignited. To facilitate recovery of stupefied mosquitoes, a white cloth was placed at the base of a tree hollow prior to fumigation, and while the gas was being generated within the hollow, basal openings to the outside were closed with canvas.

One hundred sixty-two hollow trees were fumigated. Fifty-five of these were within one mile of Mossy Pond. The other 107 hollow trees were selected at random near the margins of ten other ponds and each was fumigated only once. As one objective of the study was to determine whether or not repeated fumigation of tree hollows in the Mossy Pond area would appreciably delay the appearance of the first spring brood of *A. quadrimaculatus* at that locality, the 55 tree hollows selected for study there, which were about half the hollow trees in the Mossy Pond area, were fumigated repeatedly in 1945 (twice in January and once in March), and again in March, 1946.

Determination of the types and sizes of tree hollows utilized by anophelines as winter retreats was also an objective of the study. Consequently, all fumigated trees were numbered and identified as to species and were measured to determine their diameter breast high and the area of the opening to the hollow. Other pertinent notes were also taken, and prior to fumigation each hollow was thoroughly examined by flashlight to determine whether or not any mosquitoes were in evidence.

In December, 1944, and in January and February, 1945, hollow trees were fumigated on days following the occurrence of freezing temperature. In March, 1945, during which no freezing temperature occurred, trees were fumigated without regard to minimum temperature.

During the winter 1945–46, approximately 60 samples of emergent vegetation, floatage, filtered surface water, and plant material shaved from floating logs and from the shore were collected at ponds. At the laboratory these were placed in water baths

[2] The writer is indebted to Dr. Harold R. Dodge for identifying all specimens of culicine mosquitoes.

kept at 80°F., and each sample was examined for 1st instar larvae after three or more days in the warm bath.

Air and water temperature records were available from instruments operated by the Emory University Field Station in connection with other studies (Goodwin and Lenert 1943). At Mossy and Springfield Ponds, where the majority of the larvae were obtained, recording thermographs furnished continuous records of the surface water temperatures, the sensitive element of the thermograph in each case being submerged but within four inches of the water surface.

RESULTS

Collections of larval specimens of *Anopheles* are summarized in table 1. Collections of adult mosquitoes are analyzed in table 2.

Larvae of *A. quadrimaculatus* were not found between December 26 and March 7, 1944–45; in the winter 1945–46, December 30 was the last day larvae of *A. quadri-*

TABLE 1

*Summary of collections of anopheline larvae, winter 1944–45**

MONTH	YEAR	NUMBER OF PONDS SAMPLED	NUMBER OF COLLECTIONS MADE	TOTAL LARVAE AND PUPAE	SPECIMENS IDENTIFIED		
					A. quad.	*A. cruc.*	*A. punc.*
Dec.	1944	6(1)	15(4)	209(84)	7(3)	19(17)	24(2)
Jan.	1945	5(1)	13(3)	231(54)	0	43(23)	8(0)
Feb.	1945	12(1)	17(3)	105(7)	0	21(5)	54(0)
March	1945	9(1)	17(5)	872(573)	115(31)	279(206)	160(11)
Total.....			62(15)	1417(707)	122(34)	362(251)	246(13)

* Numbers in parentheses refer to collections and specimens from Mossy Pond. In each case the number in parentheses is included in the preceding figure which represents total collections or total specimens from all collections.

maculatus were found until March 8 when they were again collected. Although not easily found in February, larvae of *A. crucians* and *A. punctipennis* were collected during each week of the winter 1944–45.

During the period December 1, 1944 to February 23, 1945 culicine larvae were not encountered, however, a total of 76 culicine larvae was found in 18 collections made at 12 different ponds between February 23 and March 31, 1945. These were: 38 *Uranotaenia sapphirina* (O.S.), 16 *Culex* (*N.*) *apicalis* Adams, 10 *Culex* (*M.*) *erraticus* (Dyar and Knab), 6 *Aedes* (*O.*) *mitchellae* (Dyar), 5 *Aedes* (*A.*) *vexans* (Meigen), and 1 *Aedes* (*O.*) *infirmatus* (Dyar and Knab).

In addition to the collections analyzed in table 2, between February 26 and March 14, 1946, 144 hollow trees (including the 55 Mossy Pond area trees studied during the previous winter) were fumigated. No adult *A. quadrimaculatus* was obtained. Also none was obtained from 80 buildings examined March 18–20, 1946.

Except for one adult female *A. quadrimaculatus*, mosquitoes were not observed in any hollow tree prior to fumigation. Of the 162 hollow trees fumigated in the winter

TABLE 2

Summary of Winter Collections of Adult Mosquitoes by Months and by Different Collecting Methods

DATE	NUMBER AND KIND OF ADULT MOSQUITO RESTING STATIONS EXAMINED	NUMBER OF STATION EXAMINATIONS (OR FUMIGATIONS)	ADULT MOSQUITOES COLLECTED					
			Anopheline Species			Culicine Species		Others
			A. quad.	A. cruc.	A. punc.	C. erraticus	U. sapphirina	
Dec. 1–31, '44	17 Hollow Trees	17 (Fumigations)	71	0	10	14	494	
Jan. 1–31, '45	98 Hollow Trees	153 (Fumigations)	369	0	47	230	576	3 Culex (M.) peccator D. & K. 1 Culex (C.) quinquefasciatus Say
Feb. 1–28, '45	24 Hollow Trees	24 (Fumigations)	2	0	2	13	10	
Mar. 1–31, '45	89 Hollow Trees	89 (Fumigations)	16*	0	51*	16	25	2 Culex (M.) peccator 1 male Aedes infirmatus
Dec. 1–31, '44	17 Red Boxes	61	33*	0	4			1 female Culex peccator
Jan. 1–31, '45	9 Red Boxes	63	7	0	1	18 females	None	1 female Culiseta melanura (Coquillett)
Feb. 1–28, '45	16 Red Boxes	34	5	0	2			3 males Aedes vexans
Mar. 1–31, '45	17 Red Boxes	95	574*	2*	150*			
Mar. 13–14, '45	47 Buildings	47	2	11	2	2		

* Numbers followed by asterisk include one or more males; other numbers include no males

TABLE 3

Analysis of Collections of Adult Mosquitoes from the Massy Area

DATE	NUMBER AND KIND OF ADULT MOSQUITO RESTING STATIONS EXAMINED	NUMBER OF STATION EXAMINATIONS (OR FUMIGATIONS)	ADULT MOSQUITOES COLLECTED Numbers indicate females except that those followed by asterisk * include one or more males									
			Anopheline Species					Culicine Species				Others
			A. quad.		*A. punc.*		*A. cruc.* Total(	*Culex erraticus*		*U. sapphirina*		
			Total	Range	Total	Range		Total	Range	Total	Range	
Dec. 20–21, 1944	11 Hollow Trees	11 (Fumigations)	67	0–40	7	0–5	0	14	0–4	494	0–309	
Jan. 2–4, 1945	55 Hollow Trees	55 (Fumigations)	288	0–29	18	0–7	0	24	0–7	465	0–409	3 Females *Culex peccator*
Jan. 10–11, 1945	55 Hollow Trees	55 (Fumigations)	30	0–5	2	0–1	0	15	0–4	38	0–23	
Mar. 1–2, 1945	55 Hollow Trees	55 (Fumigations)	10	0–2	8	0–3	0	6	0–2	0		
Mar. 13–14, 1945	47 Farm Buildings	47	2		2		11					
Dec. 1–31, 1944	11 Red Boxes	43	33*	0–6	4	0–2	0					
Jan. 1–31, 1945	9 Red Boxes	63	7	0–2	1	0–1	0					
Feb. 1–28, 1945	9 Red Boxes	27	5	0–2	0		0					
Mar. 1–31, 1945	10 Red Boxes	62	372*	0–31*	29*	0–5*	2*					
Breakdown of March Collections from Red Boxes by Date of Collection												
Mar. 1, 1945	8 Red Boxes	8	0		0		0					
Mar. 7, 1945	8 Red Boxes	8	1*	0–1*	5	0–3	0					
Mar. 14, 1945	10 Red Boxes	10	100*	3–27*	7*	0–3*	1*					
Mar. 16, 1945	9 Red Boxes	9	139*	6*–31*	10	0–5*	0					
Mar. 21, 1945	9 Red Boxes	9	50*	1–15*	1	0–1	1					
Mar. 22, 1945	9 Red Boxes	9	28*	0–8*	0		0					
Mar. 29, 1945	9 Red Boxes	9	54*	0–12*	6*	0–3	0					

1944–45, 66 provided female specimens of *A. quadrimaculatus*, and females of *A. punctipennis* were obtained from 19. Adult females of *Culex erraticus* were represented in 52 of the collections by fumigation and *Uranotaenia sapphirina* females were present in 37.

An analysis of the collections of adult mosquitoes from the Mossy Area during the winter 1944–45 is presented in table 3.

The first complete fumigation of the 55 hollow trees in the Mossy area January 2–4, 1945 provided 288 female specimens of *A. quadrimaculatus* with 29 being the greatest number taken from any tree (table 3). The distribution of these specimens was studied to determine the sizes and types of tree hollows occupied by adult females of *A. quadrimaculatus* in the winter.

Forty-five of the 55 trees were live oaks (*Quercus virginiana* Mill.), seven were black gums (*Nyssa biflora* Walt.), two were maples (*Acer. sp.*), and one was a magnolia; preference of the adult mosquitoes for a particular species of tree was not revealed. Ten of the trees were within 50 feet of Mossy Pond while the others were distributed throughout the area; the adult mosquitoes were not concentrated in the trees closest to the pond. The trees ranged in size from a diameter breast high of 11 to 45 inches, the mean being 22.8 ± 7.2 inches (S.D.); 16 specimens were obtained from the 22 trees with a diameter breast high less than 21 inches, while 272 were obtained from the 33 larger trees. In the different trees the area of the opening to the hollow varied from 16 to 1540 square inches; analysis indicated that the mosquitoes preferred tree hollows with an opening greater than 100 square inches. The interior of twenty of the hollow trees was charred from some previous fire and the mosquitoes were somewhat more numerous in these than in those not charred.

Adult females of *Uranotaenia sapphirina*, unlike those of *A. quadrimaculatus*, were present in largest numbers in tree hollows which had small openings to the exterior. Approximately two thirds of the specimens of this species were obtained from two fumigations, 309 females in late December from a tree hollow with an exterior opening of 48 square inches and 409 females in early January from a hollow with an opening of 36 square inches. On January 31, 1945, 137 females of *Culex erraticus* were obtained from one hollow tree by fumigation; otherwise, 20 individuals constituted the greatest number taken by a single fumigation.

One larva of *A. quadrimaculatus* was reared from a sample of vegetation collected at the water line in Springfield Pond on February 14, 1946, while larvae of this species were not found in routine collections through the winter until March 8, 1946. Larvae were not obtained from the other samples of material kept in warm bath.

DISCUSSION

In a winter study of anophelines in an adjoining county, Barber *et. al.* (1924) found larvae of *A. crucians* during all winter months but no larvae of *A. punctipennis* during January and none of *A. quadrimaculatus* during January or February. The results reported here are similar (table 1) except that larvae of *A. punctipennis* were found in all winter months. The presence of anopheline larvae in the mud, etc., at the bottom of ponds, pools and streams in the winter as reported by Balfour (1928), Griffitts (1918), and Mayne (1926), was not confirmed in the current study as far as

observations in the field are concerned. However, in cold weather anopheline larvae were consistently observed at the bottom of rearing containers in an unheated laboratory.

Sulfur dioxide proved superior to hydrogen cyanide or a pyrethrum aerosol as a fumigant for obtaining adult mosquitoes from tree hollows in the winter. The initial heat activated the mosquitoes in the recesses of the tree and a toxic effect was obtained with the subsequent evolution of the gas. The gas appeared to have little or no residual effect, for adults were obtained by fumigations of the same trees on successive days. *A. quadrimaculatus* females were most numerous in hollow trees with a charred interior having a diameter breast high greater than 21 inches and an entrance opening greater than 100 square inches.

During the cold weather of January, 1945, when adult females of *A. quadrimaculatus* were difficult to locate in natural and artificial resting places, fumigation of hollow trees provided a considerable number of specimens (table 2). The tendency of females of this species to retreat to hidden and inaccessible recesses of hollow trees in cold weather may account for the paucity of specimens reported by Balfour (1928), Boyd and Weathersbee (1929), and Barber *et. al.* (1924) in their winter studies. While 288 females were obtained in the fumigation of 55 hollow trees in the Mossy Area January 2–4, 1945, subsequent fumigation of the same trees indicated a marked decline through the winter in the number of surviving specimens (table 3). This decrease in the numbers of *A. quadrimaculatus* during winter was noted by such investigators as Hinman and Hurlbut (1940), Hinman (1934, 1936), Boyd and Weathersbee (1929), and Barber *et. al.* (1924). It should be noted that the above 288 specimens obtained in early January, 1945 had survived an air temperature of 18°F. on December 15, 1944, the coldest temperature of that winter.

The results of the fumigations (table 3) show that adults of *A. quadrimaculatus* and *A. punctipennis* were generally distributed in small numbers in many trees. Barber *et. al.* (1924) found dispersion to be characteristic of overwintering *A. quadrimaculatus* females. The concentration of adults of the species reported in a fort in southern Louisiana by Hinman (1934, 1936) is probably not typical of the winter behaviour of *A. quadrimaculatus* in the southern United States, but, as suggested by King (1934), may occur only in such sub-tropical climates as are present at the extreme southern limits of the range of the species.

The intensive fumigation of hollow trees in the Mossy Area did not have any noticeable effect on the date of appearance of the spring generation of *A. quadrimaculatus* in that locality (table 3). While larvae of this species were not found in any pond during January and February, both larvae and adults increased abruptly at all ponds early in March at the time the first male of the spring generation appeared.

Hurlbut (1943) determined that from 8,976 to 12,384 degree-hours Fahrenheit (water temperature) above a developmental zero of 50°F. were required for the development of *A. quadrimaculatus* from recently deposited egg to adult. Males were not collected in the spring of 1945 until March 7, but on that date were taken at both Springfield and Mossy Ponds. By projecting backward from March 7 the appropriate number of developmental degree-hours accumulated in Mossy and Springfield Ponds (table 4), as calculated from the respective thermograph records, it should be possible

to arrive at some idea of the time deposition of the eggs which resulted in the first individuals to emerge in the spring. According to these records and Hurlbut's figure for the required (minimum) developmental degree-hours, the egg which resulted in the first male at Mossy Pond could have been deposited no later in the year than January 8, 1945, while the March 7 males at Springfield Pond must have been freshly deposited ova on or before January 2. However, Hurlbut (1943) and Huffaker (1944) found that an average daily water temperature of at least 50°F. was necessary for survival of larvae of *A. quadrimaculatus*. This temperature was not attained at Springfield Pond until February 4, 1945, and if the oviposition had taken place January 2 (as calculated above) hatching should have occurred on January 24, since the Springfield Pond thermograph record showed that by that date the minimum number of hour degrees required for hatching (1074 according to Hurlbut) had accumulated. Thus, there appears to be a contradiction in that if the ova had hatched by January 24 the mean daily water temperature would have been insufficient for the young larvae to survive, and if the ova had not hatched until February 4 there would have been an insufficient budget of degree-hours accumulated by March 7 for completion of development. However, Huffaker (1944) found that at a constant temperature *A. quadrimaculatus* developed from egg to adult more slowly than when reared at an equivalent mean temperature resulting from alternating periods of higher and lower temperature in each 24 hours, provided the daily period at the warmer temperature was of nine or less hours duration. Hurlbut's calculations were based on data from rearings at constant temperatures. Furthermore, it must be borne in mind that the

TABLE 4

Water Temperature Degree-Hour Accumulations Above 50°F. at Mossy and Springfield Ponds, Baker County, Georgia—By Weeks Calculated from the Recording Thermograph Records

WEEK ENDING	MOSSY POND ACCUMULATION OF DEGREE-HOURS (ABOVE 50°F) DURING THE WEEK	SPRINGFIELD POND ACCUMULATION OF DEGREE-HOURS (ABOVE 50°F) DURING THE WEEK
Dec. 9, 1944	No record	565
Dec. 16, 1944	No record	149
Dec. 23, 1944	No record	109
Dec. 30, 1944	No record	850
Jan. 6, 1945	No record	840
Jan. 13, 1945	118	143
Jan. 20, 1945	123	373
Jan. 27, 1945	387	584
Feb. 3, 1945	437	246
Feb. 10, 1945	366	610
Feb. 17, 1945	1248	1073
Feb. 24, 1945	1680	1796
Mar. 3, 1945	1736	2045
Mar. 10, 1945	2527	2627
Mar. 17, 1945	2134	2358
Mar. 24, 1945	2727	2765
Mar. 31, 1945	2890	2205

sensitive elements of the recording thermographs which provided the data for degree-hour accumulations in the present study were beneath the water surface, at times as much as four inches. The zone just beneath the surface film normally occupied by anopheline larvae must be more sensitive to fluctuations of temperature than zones at greater depth. The bulk of the degree-hours (above 50°F.) in February and March were accumulated in the daytime and doubtless did so more rapidly in the few millimeter zone just beneath the water surface than in the measured zone a few inches deeper. Therefore, the oviposition which resulted in the newly emerged adults of March 7 may have occurred somewhat later in the winter than indicated in the above calculations. As shown in table 4, the weekly contribution of developmental degree-hours was considerably greater during the few weeks immediately preceding March 7 than during the weeks of earlier winter. Probably there was an adequate heat budget in the few millimeter zone just beneath the water surface to permit individuals deposited as ova in late January or early February to have completed all developmental stages by March 7. The only larva of *A. quadrimaculatus* obtained from the various samples of material placed in warm bath during the winter 1945–46 was a specimen which reared from a Springfield Pond sample collected on February 14. In this second winter newly emerged adults (males) were first observed about a week later in March than in the previous year. It appears that although the population of adult females of *A. quadrimaculatus* diminishes with the progress of the winter in southwest Georgia, a sufficient number survive to provide viable ova when water temperatures have risen toward the end of winter.

The fact that adult females of *Uranotaenia sapphirina*, *Culex erraticus*, *Culex peccator*, and *Culex quinquefasciatus* were obtained in the winter (the first two in considerable numbers) by fumigation of hollow trees suggests that these species survive the winter as fertile females. Dyar (1922) was of the opinion that *Uranotaenia sapphirina* overwintered in the adult stage, and Hinman (1935) reported a large winter concentration of adults of this species in a fort in southern Louisiana. The presence of adult females of *Culex erraticus* and *Uranotaenia sapphirina* in collections by successive fumigations of the same trees indicates that the females of these species are active during the winter, although it is possible that the adults were not all removed by each fumigation.

SUMMARY

1. In southwest Georgia, larvae of *Anopheles quadrimaculatus* (Say) were not found in January or February but on theoretical grounds it was demonstrated that they were present in February at least; larvae of *Anopheles crucians* Wied. and *Anopheles punctipennis* (Say) were found in all winter months.

2. In the winter, fumigation of hollow trees by sulfur dioxide gas provided considerable numbers of adult females of *Anopheles quadrimaculatus*, *Culex* (*Melanoconion*) *erraticus* (Dyar and Knab), and *Uranotaenia sapphirina* (Osten Sacken) while in collecting by other methods adult specimens of these species were at most only rarely encountered. The fumigations provided female specimens of *Anopheles punctipennis*, *Culex* (*M.*) *peccator* Dyar and Knab, and *Culex* (*C.*) *quinquefasciatus* Say in small

numbers. All of these species are presumed to winter in South Georgia as fertile females.

3. Repeated fumigation during the winter of about half the hollow trees in a restricted area did not noticeably affect the time of appearance of the spring generation of *Anopheles quadrimaculatus* at that locality.

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OBSERVATIONS ON OVARIAN DEVELOPMENT AND FAT ACCUMULATION IN *ANOPHELES QUADRIMACULATUS* AND *ANOPHELES PUNCTIPENNIS*

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This study was initiated primarily to explore the amount of fat accumulation and the status of the ovaries in overwintering females of *Anopheles quadrimaculatus* Say in a malarious area of South Georgia. For comparative purposes, an autumn series of *A. quadrimaculatus* was examined. The study was extended to include adult females of *Anopheles punctipennis* (Say) when encountered during the winter.

MATERIALS AND METHODS

The specimens studied were collected in western Baker and eastern Early Counties, Georgia. The winter collections were made between December 1, 1944 and March 31, 1945 inclusive, and autumn collections were made in September and October, 1945. The mosquitoes were obtained by hand collecting from artificial resting places (the one foot cubical red boxes described by Goodwin, 1942) and by fumigation of hollow trees. For the fumigations sulfur dioxide gas was used in the winter, and in autumn chloroform propelled by methyl chloride was used. More detailed description of the collecting methods has been given elsewhere (Zukel 1949).

Six hundred ninety-three adult females of *A. quadrimaculatus* collected in the winter, and 319 collected in September and October were dissected. Also dissected were 240 *A. punctipennis* females collected in the winter. Dissection consisted of severing each mosquito's abdomen from its thorax and drawing out the alimentary canal in saline. The fat was then rolled out of the abdomen with a needle. Sudan II was used to stain the fat in doubtful specimens, and in some individuals osmic acid was used to differentiate the ovarian stages. On dissection of each female, the amount of fat was estimated visually and recorded as the percentage of the volume of the abdomen occupied; the empty alimentary canal serving as a standard of comparison between successive specimens.

Perry's (1912) criteria for evaluating the probable age of adult anophelines from their external appearance, Christophers' (1911) stages of ovarian development, and Rice and Mohan's (1936) classification of the degrees of ingestion and assimilation of blood were followed in describing the status of each anopheline female dissected.

Perry's schema for grading specimens was as follows:[2]

W 1 Wing well-marked and wing fringe practically complete.

W 2 Wing fairly well-marked but with wing fringe somewhat worn.

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[2] To avoid confusion with other classifications the author has inserted the symbol "W" before each of Perry's class designations.

W 3 Wing decidedly shabby and wing fringe very much worn.

W 4 Wing actually threadbare.

Christophers described in detail development of the ovaries in adult female anophelines. The stages he characterized are presented here in an abbreviated form:

Stage I Apical follicles of each ovary very small; each apical follicle with its enclosed nurse cells and "ovum" increases in size two or three times.

Stage II The appearance of yolk granules in the "ovum" (distinguishing it from the associated nurse cells within each follicle) marks the beginning of this stage. "Ovum" comes to occupy half the growing follicle.

Stage III Nucleus of "ovum" obscured by accumulation of coarse yolk granules; ovum comes to occupy about 3/4 of the follicle.

Stage IV Each apical follicle and enclosed ovum undergoes elongation, finally attaining shape of mature egg. Nurse cells have become less and less conspicuous and ovum occupies 4/5 or more of follicle.

Stage V The floats and other sculpturing of the exocorion are developed upon each egg.

According to Christophers, the folliculars tubes which make up the bulk of an ovary are each composed of a chain of follicles, the apical follicle of each tube maturing through the stages described above to produce an egg. When the mature eggs of one batch are deposited, the next follicle of each tube may mature provided additional blood is taken by the mosquito, etc. However, only freshly emerged adults which have not previously matured a batch of ova are found in Stage I; after depositing one or more batches of ova, the mosquito may be found in any stage from II to V.

Rice and Mohan's classification of adult female anophelines in relation to their blood feeding was as follows:[3]

B 1 Free from blood

B 2 Freshly ingested blood

B 3 Partially digested blood

B 4 Old and freshly ingested blood

B 5 Nearly completely digested blood

RESULTS

During the winter no specimen was found which could be assigned to either of Perry's classes W 3 or W 4, all specimens having wings relatively intact and being assignable therefore to classes W 1 and W 2.

In table 1 the relationship between blood digestion and ovarian development is summarized.

Some fat was present in all anopheline females dissected during the winter. The amount varied from an estimated 1 to 80 per cent of the volume of the abdomen. This wide variation was observed among specimens collected in each winter month and among specimens collected in September and October as well. Mosquitoes in

[3] For convenience the author has preceded the symbol for each of Rice and Mohan's classes by the letter "B".

all ovarian stages, except those in Stage B1–V which consistently contained the least amount of fat, were found to show a wide variation in the amount of fat present.

Specimens with the ovaries partly developed (Stage II) and retaining a few mature eggs as a result of incomplete oviposition were not encountered in this study. Boyd (1927) found such females among *A. quadrimaculatus* adults in North Carolina.

TABLE 1

Summary of Blood-Digestion Ovarian-Development Relationship in A. quadrimaculatus *and* A. punctipennis

CLASSIFICATION		NUMBER OF SPECIMENS									
		A. quadrimaculatus						*A. punctipennis*			
Rice-Mohan Blood Group	Ovarian Developmental Stage of Christophers	Dec. 1944	Jan. 1945	Feb. 1945	Mar. 1945	Sept. 1945	Oct. 1945	Dec. 1944	Jan. 1945	Feb. 1945	Mar. 1945
B1	I	2	0	0	21	11	34	1	0	0	7
B1	II	71	296	1	153	70	75	36	42	1	68
B1	V	0	14	1	16	7	3	3	9	1	17
B2	II	0	5	3	2	25	4	3	1	1	3
B2	III	2	17	1	41	9	21	3	6	0	13
B2	IV	1	7	0	1	0	0	3	2	0	2
B3	IV	0	5	0	10	17	12	1	2	0	3
B3	V	0	1	0	2	0	0	1	0	0	6
B5	V	0	2	0	18	15	16	0	2	1	2
Total		76	347	6	264	154	165	51	64	4	121

DISCUSSION

As anophelines with the wing fringe worn or shabby in appearance or with a few mature ova retained in the ovaries were not found it was not possible to study the relationship between deterioration of the wing and age of the mosquito. Perry (1912) in a study of *Anopheles culicifacies* in India surmised that wing deterioration was probably closely correlated with the number of times that ova had been deposited. He did not find that deterioration of the wing was correlated with egg development except that specimens in Christophers' ovarian Stage I occurred in the wing classes W 1 and W 2 only.

The presence of blood in anophelines collected during the winter indicated that *A. quadrimaculatus* and *A. punctipennis* females were active throughout the winter in this area. Specimens were not found which had taken a second blood meal while a former blood meal was being digested. Keener (1945) reported that under insectary conditions some specimens of *A. quadrimaculatus* would take a second blood meal. Separation of ingested blood into plasma and a red opaque portion as reported by de Buck *et. al.* (1932) for *A. maculipennis* was not observed in any specimen of *A. quadrimaculatus* or *A. punctipennis* examined.

The majority of the specimens collected in the winter were free of blood, with the ovaries in Stage II (table 1). Nicholson (1921) found that *A. maculipennis* usually passed the winter with the ovaries in the resting stage. A majority of specimens of

A. quadrimaculatus and *A. punctipennis* from winter collections in North Carolina contained blood or mature eggs according to Boyd and Weathersbee (1929), but in a later study, Boyd (1930) found no gravid females during the winter.

The consistent relation between blood ingestion and ovarian development recognized in other studies of anophelines was observed in *A. quadrimaculatus* and *A. punctipennis* (table 1). The presence of newly ingested blood stimulated the development of the ova which matured as the blood was nearly completely digested.

Some fat was present in all field collected anophelines, but no pattern of fat accumulation was evident between species, months of collection, or types of resting places. A variation in the amount of fat stored by females of *Anopheles quadrimaculatus* during the winter was reported by Hinman and Hurlbut (1940) in the Tennessee Valley.

SUMMARY

1. In dissections of wild adult females of *Anopheles quadrimaculatus* and *A. punctipennis* collected in South Georgia in the winter, a wide variation was found in the amount of stored fat; a similar condition was found among specimens of the former species collected in autumn.

2. A majority of both species passed the winter with the ovaries only slightly developed (Christophers' Stage II). Both species produced eggs after ingestion of a blood meal.

3. The frequent discovery of unassimilated blood in the gut of specimens of *A. quadrimaculatus* and *A. punctipennis* collected throughout the winter attests to their activity in this season.

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A SECOND YEAR'S FIELD TRIAL WITH CHLOROQUINE SUPPRESSION OF HIGH ENDEMIC MALARIA IN A PANAMANIAN VILLAGE[1]

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The village of Piña, Republic of Panama, located on the Atlantic Coast, is one of a group of similar villages selected by Elmendorf (1947) for experiments in malaria control methods by the Army School of Malariology. After a year (1945) of preparatory survey by blood smears and splenic examinations, the inhabitants of the village were offered chloroquine weekly for a period of one year (1946). A similar town, Rio Indio, located in the same area, was selected as a control village, the inhabitants being examined frequently by thick smear technique and splenic examination, but given no medication. No other control measures had been used in either of the villages. Meteorological factors were, for the purpose of this experiment, the same.

The results of these first two years of study by Elmendorf (1947) showed that suppressive chloroquine administration alone would greatly reduce the percentage of parasite-positive thick blood smears. Other field trials by Goldsmith (1947), Clark (1947), Galindo and Peruvian workers (1948), showed similar results. This present study reports the findings of the second year (1947) of suppressive medication with chloroquine.

Following the discontinuance of the Army School of Malariology, December, 1946, the people of Piña were again offered suppressive chloroquine medication under the direction of the Surgeon's Office of the United States Army Caribbean, with the cooperation of the Ministry of Health of the Republic of Panama, and at the request of the Surgeon General of the Army. Between the two programs, that reported by Elmendorf (1947) and this study, there was an interval of seven weeks during which no medication was given. The attempted control or suppression of malaria infections with chloroquine was the only phase of the original program reported by Elmendorf (1947) which was retained during this study. The same methods of drug dispensing, smear collection, and observation were continued. Examination of blood smears by the same technicians at the Gorgas Memorial Laboratory in Panama City was made possible by the director, Dr. Herbert C. Clark.

On the same day of each week, a team composed of a medical officer and one or two assistants visited the village, and dispensed chloroquine in the following dosages; adults—0.3 gram base; children (4–14)—0.15 gram base; children (under 4 years)—0.075 gram base.

[1] This work was performed under the direction of Colonel Thomas N. Page MC, formerly Medical Inspector, now Surgeon, United States Army Caribbean, Quarry Heights, Canal Zone. Sgt 1cl. Vincent D. Williams assisted in both laboratory and field phases of this work.

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Records for each individual were kept carefully on index cards, on which were recorded name, age, sex, date of each blood examination with result, date of each medication given with the dosage, type of administration, and comments on toxicity if observed. A special symbol was used to indicate treatment received at hands of medical officer. A second symbol was used to indicate medication given by the school teacher or other village official to persons not present at the time of our visit. Since personal knowledge of medication was not possible in these cases, they were classified separately. Those receiving no medication were indicated by a third symbol. Cooperation was good, since for a year (1946) these people had enjoyed the benefits of freedom from malaria symptoms. Thick blood films were made at monthly intervals for the first four months and at bi-monthly intervals for eight months thereafter, and stained and examined by the technique of Barber and Komp (1929).

The results of the surveys during 1947 in Rio Indio (the control town) and of Piña in 1945 and 1947 arranged in monthly sequence, are shown in Tables 1 and 2.

TABLE 1

Results of blood surveys by months during 1947 at Rio Indio, R. P.

MONTH	NUMBER EXAMINED	NUMBER POSITIVE	PER CENT POSITIVE	PER CENT POSITIVE *P. falciarum*	PER CENT POSITIVE *P. malariae*	PER CENT POSITIVE *P. vivax*	PER CENT POSITIVE *P. falciparum* and *P. malariae*	PER CENT POSITIVE *P. falciparum* and *P. vivax*	PER CENT POSITIVE *P. vivax* and *P. malariae*
February	40	20	50.0	40.0	40.0	5.0	5.0		10.0
March	38	21	55.2	57.0	33.0	10.0			
April	56	16	28.6	19.0	81.0				
June	58	26	44.8	46.0	42.0	4.0		4.0	4.0
August	65	48	73.8	58.0	19.0	13.0	4.0	4.0	2.0
October	59	28	47.5	43.0	46.0	11.0			
December	66	38	58.6	42.0	40.0	10.0	5.0	3.0	

Table 1 shows the results during 1947 of frequent thick film surveys at Rio Indio, the control village which received no medication.

The results of surveys during 1945 and 1947 at Piña are shown in Table 2. The administration of chloroquine as described above, was initiated at Piña on January 29, 1947.

The monthly malaria rates in Piña during 1947 (Table 2), were reduced from those in 1945, the control year, and were also much lower than in Rio Indio, the control village during 1947. Since it was obvious that the taking of one tablet at any time prior to a blood examination should not be considered adequate suppressive treatment, an arbitrary standard was established for segregating those who had not received sufficient medication. On the basis of evidence collected under the auspices of The Board for Coordination of Malarial Studies of the National Research Council, and distributed in pamphlet form by the Winthrop Chemical Company (1947), the following standard was established to distinguish treated and/or adequately treated.

All persons who had taken one or more tablets prior to any positive smear were con-

sidered treated. All persons who had taken two tablets on two consecutive weeks immediately prior to any positive smear were considered adequately treated.

All persons, whether "treated" or "untreated", are included in the monthly percentages for Piña, untreated cases examined frequently distort the percentages if

TABLE 2

Results of blood surveys by months during 1945 and 1947 at Piña, R. P.

MONTH	NUMBER EXAMINED	NUMBER POSITIVE	PER CENT POSITIVE	PER CENT POSITIVE *P. falciparum*	PER CENT POSITIVE *P. malariae*	PER CENT POSITIVE *P. vivax*	PER CENT POSITIVE *P. falciparum* and *P. malariae*	PER CENT POSITIVE *P. vivax* and *P. malariae*	PER CENT POSITIVE *P. vivax* and *P. malariae*	PER CENT POSITIVE *P. falciparum* *P. vivax* and *P. malariae*
Apr 45	142	47	33.1	75.0	4.0	17.0	2.0	2.0		
Aug 45	141	54	38.3	74.0	13.0	11.0			2.0	
Dec 45	136	92	67.6	67.0	13.0	12.0	4.0	4.0		
Jan 47	100	15	15.0	73.0	7.0	20.0				
Feb 47[1]	46	8	21.0	75.0	25.0					
Mar 47	84	12	16.7	67.0		33.0				
Apr 47	105	23	22.0	70.0	18.0	4.0	4.0			4.0
Jun 47	114	3	2.6	67.0		33.0				
Aug 47	124	3	2.4	67.0		33.0				
Oct 47	128	12	9.4	50.0	8.0	42.0				
Dec 47	178	4	2.3	100.0						

[1]Chloroquine suppressive treatment started January 29, 1947.

TABLE 3

Cumulative malaria rates at Piñā, R. P.

	1945	1947
Number examined	136	282
Number positive	98	78
Per cent positive	72.0	27.7
Number of untreated positives		40
Per cent of untreated positives		14.2
Number of treated positives[1]		38
Per cent of treated positives		13.5
Number of adequately treated positives[2]		6
Per cent of adequately treated positives		2.13

[1] "Treated" considered one or more tablets chloroquine prior to any positive smear.

[2] "Adequately treated" considered two tablets on two consecutive weeks immediately prior to any positive smear. "Adequately treated" positives are included in "Number of Treated Positives".

an evaluation of the drug alone and not a program of administration is desired. The relatively high rates in Piña during the first four months of 1947, as shown in Table 2, were attributed to two factors; (1) the January 1947 rate of 15 per cent positive was found after a lapse of seven weeks in which no medication was offered in the village; (2) the rising rates during February, March and April occurred during a time when a group of persons within the village was offered a harmless placebo

instead of chloroquine in an attempt to evaluate the relapse or reinfection rate after former suppression.

A more comprehensive picture of the results of the medication program in Piña during 1947 can be seen by comparing the cumulative malaria rates for the years 1945 and 1947 as presented in Table 3.

The cumulative figures, by considering each individual with one or more positive smear in any month during the year as a single positive, eliminate the distortion of recurrent positive cases and reduce the error inherent in a comparison of monthly rates in a village where not all inhabitants are present for each examination.

Although this work was not primarily designed to yield information on the effects

TABLE 4

A group of 24 individuals examined at Piña over a three year period

	1945	1946		1947					
	Dec	May	Aug	Jan	Mar	Jun	Aug	Oct	Dec
Number examined	24	24	24	24	24	24	24	24	24
Number positives	22	0	0	4	1	0	0	2	0
Per cent positive	91.6	0.0	0.0	16.6	4.2	0.0	0.0	8.3	0.0
Number untreated positives	22								
Per cent of untreated positives	91.6								
Number treated positives[1]				4				2	
Per cent of treated positives				16.6				8.3	
Number adequately treated positives[2]					1				
Per cent of adequately treated positives					4.2				

[1] "Treated" considered one or more tablets chloroquine prior to any positive smear.

[2] "Adequately treated" considered two tablets on two consecutive weeks immediately prior to any positive smear.

of chloroquine in suppressive doses on individuals or individual parasite infections, several attempts were made to analyze different groups within the village.

In an effort to determine the effect of suppressive chloroquine on individuals over the three year period (1945–1947), many groups were formed from the available data. Table 4 shows a significant group of 24 inhabitants of Piña who had been present for a blood examination during the control year, 1945, for 2 examinations and treatment by Elmendorf (1947) in 1946, and for 6 examinations and treatment by the writers in 1947. All members of this group received some medication in 1946 and 1947, and all had failed to receive medication for 7 weeks from December 1946 through January 1947.

Table 4 shows the dramatic response of this group to the program of suppressive chloroquine treatment. During 1945, every person in this group of 24 was positive one or more times. In December 1945, there were 22 positive smears in the 24 examined, a positive rate of 91.6 per cent. A total of 78 per cent of these positive infections was *P. falciparum* (alone or mixed); 25 per cent *P. malariae* (alone or mixed);

23 per cent *P. vivax* (alone or mixed). Single infections totaled 73 per cent and mixed infections totaled 27 per cent. Following this December survey, all persons within this group were offered suppressive chloroquine treatment by Elmendorf (1947), and on the two dates in 1946 when all were examined again, there were no positives. On the survey of January 29, 1947, after seven weeks without suppressive treatment with chloroquine, four of this group were positive for *P. falciparum*, making the malaria rate 16.6 per cent. Considering the history of this group with their high percentage positives in December 1945 and the variety of parasite species, the low percentage, found after no medication had been taken for seven weeks, is remarkable. Several questions arise. Were these positives reinfections or relapses? Why were there not more positives in the group? Was the number of parasite-infected mosquitoes reduced sufficiently by this time to lower the chance for reinfection of humans? Does chloroquine have any curative action against *P. vivax* and *P. malariae* if given in suppressive doses? Does chloroquine effect persist for 49 days after last dose? None of these questions can be answered by this work, but the fact remains that this program of chloroquine administration, even with its obvious "loopholes" in medication, did more than simply suppress malaria in this group for as long as they were on medication

When this group of 24 people was surveyed in March and April 1947, one of the group was positive for *P. falciparum* although "adequate treatment" had been given In June and August, all were negative. In October, two were positive, both *P. falciparum*. These two positives had failed to take chloroquine within two weeks prior to the occurrence of the positive smears. In December, all 24 were negative. This history is in striking contrast to that of December, 1945 when 22 were positive.

The fact that chloroquine was still an experimental drug in 1946 led Elmendorf (1947) to restrict its use to children over three years old. After the program was resumed in 1947, it was observed that many of the positive blood smears were those of children below the age of three years. When symptoms were observed, these children were given atabrine in therapeutic doses, but under this regimen, one child aged six months died of cerebral malaria in July 1947. The mother had given this child only enough of the atabrine prescribed to arrest the fever, and had given the remainder to her other children. At this time it was learned that Clark (1947) had administered chloroquine to children under four years in his experimental villages in the Gatun Lake region of Panama. Since this precedent had been set, and because the untreated children in Piña were so highly infected, it was decided in the latter part of July 1947, to offer treatment to all children regardless of age.

In a special survey, 23 July 1947, 20 children of three years or under were examined, of whom 10 (50 per cent) were positive. One quarter of an experimental 0.3 gram base tablet or 0.075 grams of chloroquine was offered each week thereafter to these children. Various methods of administration were tried. The most successful means found was crushing a quarter tablet in a metal spoon and adding cherry or cinnamon syrup to the powder to form a suspension of the drug. Although many of the younger children did not receive a full dose of the drug due to difficulty in swallowing, the results indicated that the method was practicable. Two weeks after treatment was

started, seven of this group who had been positive on the day of the first treatment were re-examined and only one remained positive, a *P. falciparum* infection. The species of parasites before treatment were one mixed *P. vivax* and *P. falciparum*, one *P. vivax* and five single *P. falciparum*.

During 1947 a total of 53 children under four years of age were examined by thick blood smear and offered treatment. Of this group, 29 were positive one or more times during the year, making the cumulative malaria percentage for the year 54.7 per cent. Removing those untreated at any time prior to any positive smear from the total, four or 14.3 per cent of those "treated" were positive. Using the criterion of two consecutive treatments prior to any positive smear for the "adequately treated" cases and removing the remaining positives from the total, two were "adequately treated" and positive, giving a percentage of 7.7. One child had received two weekly treatments. the other three weekly treatments prior to the positive smear.

A physical examination was performed on each child prior to medication, and frequent examinations were made later. Parents were questioned about any toxic symptoms, and no undesirable effect of the medication in this group was found.

A total of 78 individuals were positive one or more times during 1947 in Piña. In separating "treated" from "untreated" cases (Table 3), the first criterion used was the absence of any medication in 1946 or 1947 prior to a positive smear. In this manner, a person having had but a single dose of chloroquine, more than a year prior to the positive smear, was still regarded as a treated case. Under these conditions 40 positive cases, or 51 per cent of the total 78 positive cases for the year, had had no suppressive chloroquine medication and could be eliminated in any evaluation of the drug action on individual cases. Some of these untreated cases were the children who were not given medication until July 1947, as described above. Others were new arrivals in the village who contributed their positive blood to the total for the year and were then offered chloroquine. Making no other correction than the elimination of these untreated positives, the cumulative rate for the year 1947 was 16.6 per cent. On the basis of the afore mentioned evidence, all persons who had taken two tablets during the two weeks immediately prior to a positive blood examination were classed as "adequately treated" positive cases. Of the 38 "treated" positive cases which had had some medication, only six could fulfill the requirements of "adequately treated." Of these six cases, two were reported to have received medication from the town official and could not be considered as true drug failures since personal knowledge of their medication was lacking. The remaining four cases had been observed taking their dosage of chloroquine on both weeks prior to the occurrence of the positive smears. Of these four "break through" cases, all had been positive at some time before medication was offered. There were three *P. falciparum* infections and one *P. vivax*. Of these four positives, three had clinical symptoms at the time of the positive smear. On December 3, 1947, the *P. vivax* case again was a drug "break through" but this time positive for *P. falciparum*. No explanation of this "break through" is attempted. The ages varied from 2 to 9 years. Three were negative on all subsequent examinations with no change in the medication procedure.

The fact that there were not more positives in the village in view of the spotty

medication records of many of those consistently negative was perhaps due to the decreased transmission caused by removal of the parasite reservoir. Thus the people taking the drug may have reduced the danger of malaria for their untreated neighbors.

The species of plasmodia found on the positive smears in Piña in 1945 and 1947 and in Rio Indio in 1947, are presented in Table 5, according to the percentage of the total positive cases for the two years.

Since Table 5 shows cumulative figures, a mixed infection for the year may represent two or more single infections or actual mixed infections on the same slide. The true mixed infections may be found in Tables 1 and 2, reported by months. The cumulative percentages in Table 5 represent the total of positive subjects, regardless of history of medication, so that no conclusions with regard to the effect of chloro-

TABLE 5

Positive infections by species, given as per cent of total positives from the cumulative malaria rates for each year

SPECIES OF PARASITES	*P. falciparum*	*P. malariae*	*P. vivax*	*P. falciparum* AND *P. malariae*	*P. falciparum* AND *P. vivax*	*P. malariae* AND *P. vivax*	*P. falciparum P. malariae* AND *P. vivax*	TOTAL *P. falciparum*	TOTAL *P. vivax*	TOTAL *P. malariae*	TOTAL SINGLE	TOTAL MIXED[1]	TOTAL	TOTAL NUMBER POSITIVE
Piña 1945	61.0	9.0	7.0	11.0	9.0	1.0	1.0	83.0	18.0	22.0	76.0	22.0	99.0	98
Piña 1947	69.2	6.4	12.8	2.6	7.7	0.0	1.3	80.8	21.8	10.3	08.4	11.6	100.0	78
Rio Indio 1947	41.5	23.4	3.2	16.0	4.3	2.1	9.6	71.4	19.2	51.1	68.1	32.0	100.0	94

[1] Mixed infections for the years include actual mixed infections and combinations of single infections in the same individual at different times during the year.

quine may validly be drawn from them. It is interesting to note however, that the species distribution in Piña in 1947, a treated year, was very similar to that of the control year 1945, with the possible exception of *P. malariae*, which showed a slight decline in Piña for 1947. Rio Indio, the control village, had a higher total percentage of *P. malariae* than did Piña. *Plasmodium falciparum* led in both villages for the three years shown, having a total yearly percentage of 70 to 80 per cent, including both single and mixed infections. *Plasmodium vivax* occurred in from 18 to 21 per cent of the infections in both villages for the period under study. Of the six Suspected "break throughs", four were *P. falciparum* and two were *P. vivax*. Of the four who were known to have taken their tablets on the two weeks immediately prior to the positive smear, three were *P. falciparum* and one was *P. vivax*. No case of *P. malariae* failed to respond immediately to "adequate treatment". No case of *P. malariae* was positive on subsequent examination.

No effort was made to find histories of toxicity in the people of Piña, except in the group of children of under 4 yerars. Observation was casual on one day each week as

the subjects appeared for their medication. Headaches were complained of by two women at different times, but both were menstruating at the time of complaint and both had similar headaches during previous menstruations. Neither refused subsequent tablets. No other complaints concerning the medicine were made.

During 1946 and the first eight months of 1947, the experimental tablets of SN 7618-5 (chloroquine) were used in Piña. These were supplied by the National Research Council through the Surgeon General's Office of the Army. Various chemical houses manufactured this tablet. It was a 0.5 gram tablet, containing 0.3 grams of the active base chloroquine. Thus a single tablet supplied the necessary adult suppressive dose for one week. The surface of this tablet was durable, but unscored for a smaller dose. For the last four months of 1947, the commercial product Aralen[4] was susbstituted for the experimental tablets then unavailable. This tablet of 0.25 grams contained 0.15 gram base chloroquine or one-half the necessary adult suppressive dose, and was scored for dividing into the 0.075 gram infant suppressive dose. Physically, these tablets were less durable than the original larger experimental tablets, and had a more chalky consistency, which allowed damage due to handling, and crumbling from the high humidity of the tropical climate. It was also found less convenient to give two tablets in place of one to adults. The scoring for division, however, was an improvement over the experimental tablet. The larger, more durable tablet containing 0.3 grams base, if socred for division into quarters, would be ideal for field *type suppressive use*, particularly in tropical climates.

SUMMARY

1. The results of one year's suppressive chloroquine treatment in the village of Piña, R.P., are presented. The controls used in this study are based on malaria rates in Piña during 1945, when no treatment was given and rates in Rio Indio, R.P., during 1947, a nearby untreated village under similar meteorological conditions.

2. It is shown in this paper that malaria infections can be suppressed or possibly eradicated in native populations if chloroquine is administered regularly once each week in adequate amounts.

3. In the dosages used, observations made throughout the course of this study indicate that the drug is well tolerated by both children and adults, no toxic symptoms being noted.

4. A total of 282 persons examined one or more times during 1947 gave a cumulative positive parasite rate of 27.7 per cent. If "untreated" and "treated" positive cases are eliminated from consideration, the cumulative parasite rate in these 282 persons was reduced to 2.1 per cent at the end of 1947. Cumulative rates and "untreated" and "treated" positives are defined.

5. Records of suppressive chloroquine administered consistently to a special group of 24 persons, all of which were positive for malaria at some time during 1945, are given for both 1946 an 1947. At the close of the year 1947, every member of this group was negative. Similar results may be expected when suppressive chloroquine is administered to a well disciplined military unit.

[4] Aralen. Brand of Chloroquine Diphosphate, known also as SN 7618-5, Winthrop Chemical Company, Inc., New York. Army-Navy Stock No. 1-139-300.

6. A group of 20 children was examined in July 1947, ten of which were positive. Seven of this group were re-examined two weeks later after two doses (0.075 gram base) of suppressive chloroquine, ony one remained positive.

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